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Kristine Nicole Brazin
*Iowa State University*

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# Proline *cis/trans* isomerization regulates a T cell specific tyrosine kinase

by

Kristine Nicole Brazin

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Program of Study Committee:
Amy H. Andreotti, Major Professor
Drena Dobbs
Mark Hargrove
Richard Honzatko
F. Anderson Norris

Iowa State University

Ames, Iowa

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# ABSTRACT

This dissertation examines the role of protein-protein interactions that regulate the conformation and function of the Tec family kinase, interleukin-2 tyrosine kinase (Itk). Tec family kinases are expressed in hematopoetic cells and modulate intracellular signaling cascades in response to external stimuli. The domain structure of Tec family members contains the conserved SH3, SH2, and catalytic domains common to many kinase families, yet they are distinguishable by the presence of a unique N-terminal sequence. The mechanism by which Itk is regulated is not well understood. Both nuclear magnetic resonance spectroscopy and functional assays were used to elucidate a novel regulatory mechanism for Itk in the work presented in this dissertation. These studies demonstrate that the Itk SH2 domain adopts two distinct conformations in solution that are in slow exchange. The observed conformational heterogeneity is due to proline *cis/trans* isomerization around a single prolyl imide bond, generating a *cis* proline-containing conformer and a *trans* proline-containing conformer. Each conformer displays unique ligand binding properties. The *trans* conformer preferentially binds phosphotyrosine-containing ligands, whereas the *cis* conformer is required for a novel intermolecular interaction with the Itk SH3 domain. This SH3-SH2 self-association interaction is mediated by the conserved aromatic ligand binding pocket on the Itk SH3 domain and a newly defined surface on the Itk SH2 domain.

Proline *cis/trans* isomerization is not only an important switch in regulating ligand binding, we also observe that the conformationally heterogenous proline residue is required for recognition of Itk as a substrate for the peptidyl-prolyl isomerase, cyclophilin A. Cyclophilin A accelerates the interconversion between the *cis* and *trans* Itk SH2 domain

conformers. Furthermore, both *in vitro* and *in vivo* cellular experiments reveal that cyclophilin A inhibits Itk kinase activity. This observation allows us to propose a mechanistic model for a completely new mode of kinase regulation and reveals a cellular role for cyclophilin A in T cell signaling. In sum, this dissertation provides molecular details about the structure of the Tec family kinase, Itk, and the functional implications of these results.

# CHAPTER 1. GENERAL INTRODUCTION

## Literature Review

Cellular signal transduction events are exceedingly dynamic, with countless regulatory pathways monitoring responses to external and internal stimuli. Protein phosphorylation and dephosphorylation, through the enzymatic activity of protein kinases and protein phosphatases respectively, can result in significant structural rearrangements that alter a protein's conformation, ultimately regulating its biochemical activity (1). Thus, protein phosphorylation acts as a molecular switch in controlling cellular signaling pathways. Several disease states, such as autoimmune disorders, cancer, and immunodeficiency disorders, are accompanied by defective intracellular signaling pathways. These defects often result from mutations in genes that encode for protein kinases. For example, chronic myelogenous leukemia results from the recombination of two genes, one of which encodes for a protein kinase, that produce a chimeric signaling molecule displaying uncontrolled tyrosine kinase activity (2, 3). The importance of protein phosphorylation in regulating cellular processes cannot be overstated. Upon completion of the human genome, approximately 3% of the genes are known to encode for protein kinases (4); however, the molecular mechanisms by which many of these enzymes are regulated are not well understood.

**Protein-tyrosine Kinases.** Protein-tyrosine phosphorylation is a reversible modification that can regulate both the conformation and function of proteins by switching

them between active and inactive states. Therefore, protein tyrosine kinases (PTKs) are critical for the regulation of cellular processes. There are two families of protein-tyrosine kinases: the transmembrane receptor family and the cytosolic nonreceptor family (5, 6). Both kinase families are necessary for mediating cellular signaling pathways. Although receptor and nonreceptor PTKs differ significantly in their noncatalytic domain structure, the catalytic domain is highly conserved between both families. The catalytic domain consists of two lobes: a small N-terminal lobe that is required for ATP binding, and a larger C-terminal lobe that is primarily involved in catalysis (7, 8). A key aspect to the regulation of PTKs is the phosphorylation of residue(s) in the region between the two lobes of the kinase domain, known as the activation segment. Phosphorylation of the activation segment results in conformational changes in the nucleotide binding site and catalytic site that allow for the correct orientation of ATP and substrate that is required for catalysis.

**Src Family Nonreceptor Protein-tyrosine Kinases.** Currently, the approximately 30 known nonreceptor PTKs have been grouped into five distinct families based on their noncatalytic domain sequences (9). The largest family of nonreceptor PTKs is the Src family, named after the src oncogene of the Rous sarcoma virus (10). The Src family consists of 9 members: Src, Lck, Fgr, Fyn, Blk, Hck, Lyn, Yes, and Yrk (11). In addition to the catalytic domain, Src kinases contain a unique N-terminal region, two noncatalytic domains: Src homology 3 (SH3) and Src homology 2 (SH2), and a C-terminal tail. The attachment of myristate, a $C_{14}$ fatty acid, to the N-terminal region is required for membrane localization

(12). The SH3 and SH2 regulatory domains mediate protein-protein interactions through their well characterized ligand binding sites. SH3 domains are known to bind proline-rich sequences that contain the PXXP motif, whereas SH2 domains bind phosphotyrosine-containing sequences (13, 14).

The regulatory mechanisms of the Src family PTKs have been defined by biochemical assays and high-resolution crystal structures (15-17). Enzymatic activity and substrate binding is mediated by two intramolecular interactions involving the SH3 and SH2 domains. Phosphorylation of Tyr527 (c-Src) in the C-terminal tail by the c-Src kinase (Csk) generates a phosphotyrosine-containing ligand that binds intramolecularly to the Src SH2 domain. The SH3 domain interacts intramolecularly with the linker region between the SH2 domain and the N-terminal kinase lobe, which adopts a polyproline type II helix. The intramolecular interactions mediated by both the SH2 and SH3 domains are critical for maintaining an inactive kinase. The contacts mediated by the regulatory domains alter the conformation of the kinase active site, which results in stabilization of an inactive state by displacing helix αC in the N-terminal kinase lobe, subsequently reorienting important ion pairs in the catalytic site, and by positioning the activation segment to block access to the catalytic site. Displacement of either intramolecular interaction through SH3 or SH2 domain ligand binding interactions with target proteins or dephosphorylation of Tyr527 in the C-terminal tail will result in autophosphorylation in trans of Tyr416 (c-Src) in the activation segment. Phosphorylation of Tyr416 results in a conformational switch in the residues near the active site, causing a restructuring of the activation segment and the movement of helix αC toward

the active site, thus allowing for a regain of catalytic activity. Mutations that disrupt either the SH3 or SH2 intramolecular interaction result in a constitutively active kinase and lead to uncontrolled cell growth.

**Tec Family Nonreceptor Protein-tyrosine Kinases.** The Tec family, which is comprised of five members, Tec, Btk, Itk, Rlk/Txk, and Bmx, is the second largest nonreceptor PTK family (18). Tec and Bmx are expressed in both hematopoetic and non-hematopoetic cells. Btk, Itk, and Rlk/Txk are expressed only in hematopoetic cells, and are known to participate in lymphocyte signaling cascades. Only Btk and Tec are expressed within B cells; however, T cells primarily express Itk, Rlk/Txk, and Tec. To date, Btk is the only Tec family kinase to be associated with a disease. Mutations or deletions in the Btk gene result in a hereditary immunological disorder, X-linked agammaglobulinemia in humans (XLA) (19, 20). Individuals with XLA have a severely compromised immune system, characterized by a lack of mature circulating B cells, thereby emphasizing the importance of Btk in B cell development.

The Tec kinase family shares considerable sequence similarity to the Src family of PTKs within the SH3, SH2 and kinase domains. However, the Tec family differs from the Src kinases in both the amino-terminal and carboxy-terminal regions. Tec kinases lack the amino-terminal myristolylation sequence and the regulatory tyrosine residue in the carboxy-terminal tail. Tec family kinases, with the exception of Rlk/Txk, contain an extensive amino-terminal region that includes a pleckstrin homology domain (PH) and a Tec homology domain

(TH). Rlk/Txk lacks both a PH and TH domain, yet has a unique amino-terminal region containing a palmitoylated cysteine-rich region (21).

The Tec family kinases are the only nonreceptor PTKs to contain a PH domain. In contrast to the constitutive membrane association of Src PTK family, the Tec kinases are able to associate transiently with the plasma membrane through binding of phospholipids, including phosphatidylinositol (3,4,5)-triphosphate ($PIP_3$), a product of phosphoinositide 3-kinase (PI3K), to the PH domain (22, 23). All members of the Tec kinases are predominately located within the cytoplasm of resting cells. Activation of Tec kinases occurs in response to stimulation of various cell-surface receptors, such as antigen receptors, growth factor receptors, G-protein coupled receptors, cytokine receptors, and integrin adhesion receptors (18, 23-26). Cell-surface receptor stimulation results in Tec family kinase membrane association, which has been shown to be required for activation. Indeed, a gain-of-function mutation in the Btk PH domain (E41K) generates an increased binding affinity for phosphatidylinositol (1,3,4,5)-tetraphosphate compared to the wildtype protein, thus resulting in an increase in membrane association and up regulation (27). Mutations in the Btk PH domain that result in a decrease or inability to bind PI3K phopholipid products, e.g. R28C, have been implicated in the impaired B cell development observed in mice with murine X-linked immunodeficiency (xid), a milder form of XLA (27, 28). Therefore, Tec family kinase activation (excluding Rlk/Txk) is dependent upon PI3K activity.

It remains unclear why membrane association results in increased Tec family kinase activity. It has been proposed that membrane association may recruit the Tec family kinases

to distinct membrane compartments where they can participate in macromolecular complexes with signaling partners such as members of the Src family of PTKs. Tec family kinases contain the conserved tyrosine phosphorylation site in the activation segment within the kinase domain, and phosphorylation of this tyrosine residue by Src family kinases results in the activation of Tec family kinases upon membrane association (29-32).

Membrane association may also increase the local concentration of Tec kinases, thus leading to increases in activity through homodimerization. Three different Btk PH domain mutants bound to phosphatidylinositol (1,3,4,5)-tetraphosphate were all observed to dimerize through the same interface (33). PH domain mediated dimerizaton may be required for Tec family kinase activation and interaction with target proteins. Although the Tec family kinases are thought to be activated by Src family kinases *in vivo*, Tec family kinases are able to directly phosphorylate the tyrosine residue in the activation segment in the absence of Src family PTKs (34). Autophosphorylation of this tyrosine residue in the activation segment may be facilitated through dimerization. The Tec family kinases also contain a tyrosine phosphorylation site within the SH3 domain. This site becomes autophosphorylated following phosphorylation of the tyrosine residue in the activation segment in the kinase domain, and leads to the subsequent activation of Tec family kinases (32, 34, 35). Although it remains to be determined if autophosphorylation of the SH3 domain in a full-length Tec kinase occurs in cis or in trans, Btk was able to phosphorylate its own SH3 domain in trans (34). Thus, similar to receptor PTKs, dimerization as an activation mechanism may play a role in Tec family nonreceptor PTK signaling cascades.

Within the Tec family kinases, the TH domain is located adjacent to the membrane translocating PH domain (36). A highly conserved region in the amino-terminal portion of the TH domain, referred to as the Btk motif, contains a zinc-binding motif. Missense mutations involving a conserved cyteine pair necessary for $Zn^{+2}$ coordination within the Btk motif have also been associated with XLA (37). Disruption of the zinc-binding motif is thought to alter the protein's stability and fold. In fact, the presence of the TH domain was essential for obtaining large quantities of recombinantly expressed soluble PH domain needed for crystallization (38). In the crystal structure, the Btk motif was shown to pack against the PH domain, forming an extended loop stabilized by the presence of a zinc ion. Therefore, mutations that disrupt $Zn^{+2}$ coordination would not only affect the fold of the TH domain but of the PH domain as well.

The C-terminal portion of the TH domain is characterized by the presence of a proline-rich region containing the PXXP consensus sequence known to bind SH3 domains. Rlk/Txk contains this proline-rich region despite lacking a conventional TH domain. The SH3 domains of Src family kinases; Fyn, Lyn, and Hck, have been shown to directly bind the proline-rich region of Btk and Rlk/Txk *in vitro* (39, 40). In addition, the proline-rich region of Tec was shown to mediate an interaction with Lyn both *in vitro* and *in vivo* (41). This interaction with Src PTKs may promote phosphorylation of the tyrosine residue in the activation segment of the Tec and therefore result in Tec kinase activation.

Btk and Tec contain two PXXP motifs within the proline-rich, amino-terminal portion of the TH domain. The N-terminal PXXP motif in both Btk and Tec binds

intramolecularly to its adjacent SH3 domain (42, 43). Whereas, the C-terminal PXXP motif participates in a higher affinity intermolecular SH3 domain binding interaction, resulting in homodimerization (42-44). Itk and Rlk/Txk contain one PXXP motif within the TH domain. The Itk PXXP motif is homologous to the N-terminal PXXP motif in Btk and Tec, and likewise binds intramolecularly to its adjacent SH3 domain (45). This Itk proline-SH3 domain intramolecular interaction has been proposed to regulate the accessibility of the SH3 ligand binding site in order to maintain an inactive/closed kinase. The PXXP motif in Rlk/Txk appears to be homologous to the C-terminal PXXP motif in Btk and Tec, thus one would predict that the Rlk/Txk PXXP motif would bind intermolecularly to a SH3 domain in a neighboring Rlk/Txk molecule. Perhaps both intramolecular and intermolecular proline-SH3 domain interactions are necessary for regulating Tec family kinase activity. Similar to the intramolecular regulatory interaction between the Src SH3 domain and the kinase linker, the intramolecular proline-SH3 domain interactions observed for Tec, Btk, and Itk may prevent homodimerization and/or binding of other SH3 domain containing proteins that may lead to activation. Although the intermolecular proline-SH3 domain interaction in Btk or Tec would also prevent interactions with other proteins, this interaction would promote homodimerization, in which the activation state remains unknown.

Autophosphorylation of the tyrosine residue in the SH3 domain (Y223 in Btk) would likely disrupt either the intermolecular or intramolecular proline-SH3 domain interaction. Inspection of the Itk, Btk, and Tec SH3 domain solution structures reveals that Y223 is located within the conserved aromatic ligand binding pocket (42, 45, 46). Y223

autophosphorylation appears to alter the ligand binding properties of the Btk SH3 domain (34). Phosphorylation of the Btk SH3 domain abolishes an interaction with the proline-rich region of the Wiskott-Aldrich syndrome protein (WASP), yet generates a proline independent high affinity interaction with the carboxy-terminal region of the spleen tyrosine kinase (Syk). In additon to the generation of novel SH3 domain ligand binding capacities, autophosphorylation may be a means of disengaging regulatory interactions mediated by the SH3 domain. The SH3 domain appears to affect the function of the Tec kinases through binding interactions with other proteins. For example, Itk becomes highly phosphoryated in response to SH3 domain binding to a proline-rich peptide originating from the costimulatory T cell receptor, CD28 (47-50). As well, mutation of Y223 or deletion of the SH3 domain in the upregulated Btk E41K mutant increased the transforming potential of Btk without affecting the kinase activity (31). Thus, the SH3 domain appears to play a regulatory role in modulating Tec family kinase function through protein-protein interactions analogous to the role of the SH3 domain in the Src PTK family.

The SH2 domain, located adjacent to the SH3 domain, may also be involved in regulating the function of Tec family kinases. Based on molecular models and ligand binding assays, the SH2 domains of the Tec family kinases appear to bind phosphotyrosine-containing sequences and adopt the characteristic SH2 domain fold, consisting of a central β-sheet flanked by two α-helices (51-54). The SH2 domains within the Tec family kinases have been shown to be required for interactions with adaptor proteins at the membrane. These SH2 mediated interactions may localize the Tec family kinases to a specific membrane

environment for participation in macromolecular complexes, which provide the scaffold required for phosphorylation and subsequent kinase activation. It has been observed that the level of Itk phosphorylation upon anti-CD3 receptor stimulation in Jurkat T cells is unaffected by the expression of a kinase-dead Itk mutant (K390R) or deletion of the Itk SH3 domain. In contrast, Itk fails to become highly phosphorylated when the Itk SH2 domain has been deleted or when the phosphotyrosine binding pocket has been mutated (55). The SH2 domain phosphotyrosine pocket mutation in Itk is thought to disrupt a phosphotyrosine-dependent interaction with the adaptor protein linker of activated T cells (LAT), and correlates with an absence of Itk phosphorylation and activation. The Btk SH2 domain also seems to be important for regulating function. Based on a model of the Btk SH2 domain, a series of mutants have been identified in XLA patients that are proposed to be located in the phosphotyrosine binding pocket (53, 54). Subsequent analysis of the Btk SH2 domain mutants using circular dichroism and surface plasmon resonance showed that the mutations decrease the binding affinity for phosphotyrosine-containing ligands. Several of these mutations affected the overall fold and stability of the SH2 domain. Therefore, the SH2 domain may be necessary for participation in intermolecular interactions necessary for localization of the Tec family kinases and activation.

As stated previously, contacts required for maintaining an inactive Src kinase are due to the formation of an intramolecular interaction between the SH3 domain and the linker region between the SH2 domain and the kinase domain. Unlike the Src family of PTKs, a direct interaction between the regulatory domains and the catalytic domain of the Tec kinases

does not appear to be neceassry in order to maintain an inactive state prior to phosphorylation of the the tyrosine residue in the activation segment. A crystal structure of the unphosphorylated/inactive form of the Btk kinase domain has been completed (56). The Btk kinase domain is proposed to maintain an inactive conformation through interactions between residues in helix αC in the N-terminal kinase lobe and residues in the activation segment, e.g. hydrogen-bonded pair Glu-445/Arg-544. Such interactions prevent the formation of a critical interaction between Glu-445 and Lys-430 in the active site, which would reorient helix αC and residues in the activation segment, allowing for a regain of catalytic activity. Phosphorylation of the tyrosine residue (Tyr-551) in the activation segment is proposed to alleviate interactions that maintain the inactive conformation of the catalytic site. Phosphorylation of Tyr-551 may result in an interaction between Arg-544 and the phosphate group of pTyr-551, thereby allowing Glu-445 to be in a postion to interact with Lys-430. Formation of the Glu-445/Lys-430 interaction would re-position helix αC and the activation segment, hence activating the catalytic site.

Activation of the kinase domain leads to autophosphorylation of the tyrosine residue in the SH3 domain, the second requirement for a fully active Tec family kinase. To date, little is known about physiological Tec family kinase substrates once activated. Analogous to many non-receptor PTKs, the Tec kinases appear to phosphorylate tyrosine-containing sequences that are ligands for their own SH2 domains or closely related SH2 domains (57). For example, Tec specifically phosphorylates a tyrosine residue in Dok-1. In turn, phosphorylated Dok-1 binds to the Tec SH2 domain, and this association results in

hyperphosphorylation of Dok-1 (58, 59). Likewise, Rlk/Txk has been shown to phosphorylate the tyrosine-containing sequence YEPP in the 76kD SH2-domain-containing leukocyte protein (Slp-76), following which, this phosphotyrosine-containing sequence of Slp-76 acts as a ligand for the Itk SH2 domain (52, 60).

The presence of four different regulatory domains, e.g. PH, TH, SH3, and SH2, enable the Tec family kinases to participate in a multitude of protein-protein interactions. These protein-protein interactions are important for controlling Tec family kinase activity, and are required for their role in mediating cellular signaling pathways. The Tec family kinases participate in a varitey of signaling pathways emanating from a number of different cell surface receptors. The T cell specific Tec family member, Itk, is essential for mediating signaling events necessary for T cell activation in response to antigen receptor stimulation

**T cell Activation.** One aspect of the immune system required for the elimination of foreign pathogens is T cell activation. An antigen-presenting cell (APC), displaying a major histocompatibility complex (MHC) bound to a foreign peptide on its surface, is able to attract and bind to a circulating T cell through its T cell receptor (TCR), thereby initiating T cell activation. Once the TCR-APC interaction occurs, TCR signaling follows within seconds, resulting in protein clustering and rapid increases in intracellular protein phosphorylation levels (61-63). In addition to the TCR, other molecules on the surface of the T cell participate in activation by functioning as co-receptors. Co-receptors are able to increase the affinity of the interaction with either the antigen or the APC, or activate separate signal

transduction events that affect the cellular response. Signaling events during T cell activation result in the secretion of cytokines that regulate the intensity and duration of the T cell immune response. The cytokine interleukin-2 (IL-2) is an autocrine growth factor produced only upon T cell activation, primarily by T cells expressing the surface antigen CD4 (64). Biological activities of IL-2, such as regulation of T cell proliferation and progression through the cell cycle, are mediated by the IL-2 membrane receptor that is expressed almost exclusively on the surface of activated T cells (65, 66). Protein phosphorylation and more specifically protein-tyrosine phosphorylation is critical for the initiation of cellular responses that lead to both the production of IL-2 and the IL-2 receptor.

The TCR lacks intrinsic tyrosine kinase activity; however, the T cell co-receptor CD4 contains a cytoplasmic domain that associates with the Src family nonreceptor PTK lymphoid cell kinase (Lck) following T cell activation (67, 68). Upon association with CD4, Lck mediates post-receptor signal transduction events by phosphorylating the TCR cytoplasmic domain sequences termed immunoreceptor tyrosine-based activation motifs (ITAM) (69, 70). ITAMs are approximately 15 residue sequences that contain two tyrosine residues with the consensus sequence YxxL (x) $_{6\text{-}8}$YxxL. Tyrosine phosphorylation of ITAMs initiates an activation cascade dependent upon PTK activity. In fact, inhibitors of PTK activity are able to disrupt both early and late events during T cell activation, such as increases in intracellular calcium levels, cytokine secretion, and T cell proliferation (71). Therefore, PTKs play a critical role in T cell activation, where nonreceptor PTKs are

essential for translating receptor signaling events in the T cell that ultimately lead to the expression of IL-2.

**The Role of the Tec Family Kinase Itk in T cell Activation.** The T cell specific Tec family kinase, interleukin-2 tyrosine kinase (Itk), is critical for mediating antigen-receptor signaling pathways, and is therefore required for T cell activation (72, 73). The importance of Itk in antigen-receptor signaling pathways has emerged through experiments on mice lacking Itk (74-78). Itk deficient mice display several defects, among which include reduced IL-2 production and impaired proliferative responses following T cell receptor stimulation.

Early T cell receptor events are unaffected by the lack of Itk, however; intermediate events are impaired that result in reduced IL-2 production and subsequently affect T cell proliferation. Immediately upon T cell receptor stimulation, Lck phosphorylates the T cell receptor ITAM motifs and Itk. Phosphorylation of ITAMs recruits the 70kD zeta chain associated protein (Zap-70) to the T cell receptor zeta chain, which leads to Zap-70 phosphorylation and activation. In turn, Zap-70 then phosphorylates LAT, and then Itk associates with phosphorylated LAT through the SH2 domain and Itk is then able to phosphorylate phospholipase Cγ1 (PLC γ1) (70, 79, 80). T cell receptor phosphorylation and Zap-70 levels of phosphorylation remain unaffected by the lack of Itk (75). However, the level of (PLC γ1) phosphorylation, and consequently activation, is decreased in Itk knockout mice, consistent with the observation that the level of PLC γ1 phosphorylation parallels the level of Itk phosphorylation. Activated PLC γ1 hydrolyzes membrane bound

phosphatidylinositol (4,5)-bisphosphate (PIP2) to produce diacylglycerol (DAG) and inositol (3,4,5)-trisphosphate (IP3) (81). IP3 then binds to IP3-gated channels on the endoplasmic reticulum (ER) and releases calcium stores from the lumen of the ER. Emptying of these stores causes the plasma membrane calcium channels to open, resulting in a sustained calcium flux that leads to the translocation of the transcription factor nuclear factor of activated T cells (NFAT). Once in the nucleus, NFAT binds to the IL-2 promotor region and activates transcription. Consistent with the role of Itk activating PLC $\gamma$1, a decrease in calcium elevation and NFAT translocation is observed after T cell receptor stimulation in Itk deficient mice, and correlates with a decrease in IL-2 promoter activity (76, 82). Thus, Itk appears to be necessary to generate fully phosphorylated and activated PLC $\gamma$1, following which IL-2 is produced and T cell proliferation is intiated upon binding of IL-2 to the IL-2 receptor.

The distinct roles of Itk in regulating T cell activation and other cellular processes are only beginning to emerge. The signaling complexes that Itk participates in, and its mechanism of regulation are not well understood. As one would expect, based on the number of different regulatory domains and the multitude of interactions with various signaling molecules, Itk appears to integrate numerous pathways emanating from a variety of cell-surface receptors. Continued use of genetic, biochemical and structural approaches will significantly advance our understanding of Itk, thus clearly defining its function in mediating important cellular processes of the immune system.

**Dissertation Organization**

Chapter 1 of this dissertation contains a review of the literature. Chapters 2 and 3 have been published in peer-reviewed journals. Chapter 4 has been submitted for publication, and has therefore been formatted according to the preferences of the journal. As first author, I performed all of the experiments described in Chapters 2-4, with the exception of figure 2 in Chapter 3, in which Dr. Robert J. Mallis calculated the SH2 domain surface structures. In addtion, Chapters 2-4 benefited greatly from discussions with Dr. Amy H. Andreotti, Dr. D. Bruce Fulton, and Dr. Robert J. Mallis. Chapter 5 provides a general summary and conclusions of Chapters 2-4.

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# CHAPTER 2. A Specific Intermolecular Association Between the Regulatory Domains of a Tec Family Kinase

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Kristine N. Brazin, D. Bruce Fulton and Amy H. Andreotti

## Summary

Interleukin-2 tyrosine kinase, Itk, is a T-cell specific tyrosine kinase of the Tec family. We have examined a novel intermolecular interaction between the SH3 and SH2 domains of Itk. In addition to the interaction between the isolated domains, we have found that the dual SH3/SH2 domain-containing fragment of Itk self-associates in a specific manner in solution. Tec family members contain the SH3, SH2 and catalytic domains common to many kinase families yet are distinguished by a unique amino-terminal sequence, which contains a proline-rich stretch. Previous work has identified an intramolecular regulatory association between the proline-rich region and the adjacent SH3 domain of Itk. The intermolecular interaction between the SH3 and SH2 domains of Itk that we now describe provides a possible mechanism for displacement of this intramolecular regulatory sequence, a step that may be required for full Tec kinase activation. Additionally, localization of the interacting surfaces on both the SH3 and SH2 domains by chemical shift mapping has provided information about the molecular details of this recognition event. The interaction involves the conserved aromatic binding pocket of the SH3 domain and a newly defined binding surface on the SH2 domain. The interacting residues on the SH2 domain do not

conform to the consensus motif for an SH3 proline-rich ligand. Interestingly, we note a striking correlation between the SH2 residues that mediate this interaction and those residues that, when mutated in the Tec family member Btk, cause the hereditary immune disorder, X-linked aggamaglobulinemia (XLA).

**Introduction**

The Tec family non-receptor tyrosine kinases (Tec, Btk, Itk, Bmx and Rlk) participate in intracellular signaling events that are initiated by stimulation of surface receptors on hematopoetic cells (Bolen, 1995; reviewed by Mano, 1999). Bruton's tyrosine kinase, Btk (Vetrie *et al.*, 1993; Tsukada *et al.*, 1993) and Interleukin-2 tyrosine kinase, Itk (Heyeck & Berg, 1993; Yamada *et al.*, 1993; Siliciano *et al.*, 1992) are important members of the B cell and T cell receptor signaling pathways, respectively. Mutations in the Btk gene cause the severe hereditary immunological disorders, X-linked agammaglobulinemia (XLA) in humans and X chromosome-linked immunodeficiency (Xid) in mice (Vetrie *et al.*, 1993; Tsukada *et al.*, 1993; Rawlings *et al.*, 1993; Thomas *et al.*, 1993). The link between alterations in the Btk sequence and the occurrence of these diseases emphasizes the importance of this family of protein kinases in achieving a proper immune response. The details of the signaling pathways that the Tec kinases participate in and the mechanism by which these kinases are regulated are not well understood. In contrast, the regulatory mechanism of the structurally related Src kinases has recently been clearly defined by high resolution crystal structures of two family members (Xu *et al.* 1997; Sicheri *et al.* 1997).

Substrate binding and enzymatic activity of the Src kinases are controlled by intramolecular interactions mediated by both the Src homology 2 (SH2) and Src homology 3 (SH3) domains (Courtneidge, 1985; Cooper, 1993; Hunter, 1987).

The Src and Tec kinase families share sequence homology in the SH3, SH2 and the catalytic tyrosine kinase domains yet display distinguishing characteristics in the carboxy- and amino-terminal regions. The C-terminal regulatory phosphorylation site in the Src kinases is absent in the Tec kinases; however, the Tec kinases contain an extensive amino terminal region made up of a Pleckstrin homology (PH) domain (Lemmon *et al.*, 1996) and a Tec homology (TH) domain (Vihinen, 1994) not present in the Src kinases. The unique amino terminal TH domain of the Tec family members contains a proline rich sequence that participates in an intramolecular interaction with the downstream SH3 domain in Itk (Andreotti *et al.*, 1997). Formation of the intramolecular SH3-proline complex within Itk regulates binding of other protein ligands and may therefore be implicated in controlling the activity of Tec family kinases (Andreotti, 1997).

Exogenous ligands have been identified that associate with the SH3 domain of Src kinases leading to displacement of the intramolecular SH3 and SH2 ligands and ultimately Src kinase activation (Moarefi, 1997). While the mechanism of Tec kinase activation is not as well understood, ligands have been identified that may potentiate kinase activity by binding to the non-catalytic domains of Tec (Bence *et al.*, 1997; Bunnell *et al.*, 1996; Bunnell *et al.*, 2000). There is mounting evidence that Itk activation is mediated in part by association with the signaling scaffold protein, Lat, which may serve to co-localize Itk with its activation

partners (Shan & Wange, 1999). It has been proposed that multivalent interactions between Itk and other co-localized signaling proteins may serve to disrupt the intramolecular proline-SH3 interaction leading to concomitant activation steps (Andreotti *et al.*, 1997; Su *et al.*, 1999; Schneider *et al.*, 2000; Bunnell *et al.*, 2000). Another line of evidence supporting the notion that displacement of the intramolecular SH3-mediated interaction is required for full Tec kinase activity is that autophosphorylation on a tyrosine residue in the SH3 ligand-binding pocket of Btk correlates with Btk kinase activation (Park *et al.*, 1996; Nisitani, 1999; Wahl, 1997). This phosphorylation event would most likely require prior removal of the intramolecular proline ligand from the SH3 binding pocket. We have now identified a novel interaction involving the SH3 and SH2 domains of Itk that may, in the proper cellular context, play a role in Itk activation by displacing the putative regulatory proline sequence from the SH3 binding pocket.

The structures and binding specificities of the two ubiquitous adaptor modules, SH2 and SH3, have been extensively investigated and are well understood (reviewed by Kuriyan & Cowburn, 1997). SH2 domains consist of a central β-pleated sheet flanked by two α-helices and bind to phosphotyrosine(pTyr)-containing sequences (for a secondary structure map of the SH2 domain see Figure 4). Phosphopeptide binding is mediated through a 'two-prong' mechanism that involves two well-defined pockets on the surface of the SH2 domain. The phosphotyrosine binding pocket of the SH2 domain consists of three conserved basic residues that interact with the phosphotyrosine sidechain through hydrogen bonds, hydrophobic and electrostatic contacts. In addition, a more variable specificity pocket on the

SH2 domain binds residues C-terminal to the pTyr. SH3 domains are made up of two perpendicular antiparallel β-sheets and are very often found adjacent to SH2 domains in signaling proteins. The SH3 adaptor module recognizes proline-rich sites that adopt a left-handed polyproline type II (PPII) helix conformation. The ligand binding site on the SH3 domain is comprised of conserved aromatic residues. Additionally, there are two variable regions, named the RT and n-src loops, that participate in ligand binding. To date, almost all SH3 ligands identified are short contiguous sequences that contain a PXXP consensus motif (Chen *et al.*, 1993). Most SH3 domains contain an acidic residue in the RT loop that forms a critical electrostatic interaction with a basic residue (arginine or lysine) adjacent to the PXXP motif in the ligand. Formation of this salt bridge determines the binding orientation of the polyproline peptide; two opposite binding orientations have been characterized (Feng *et al.*, 1994).

There are notable exceptions to these well-characterized SH2 and SH3 consensus ligands. Phosphotyrosine-independent SH2 mediated interactions have been observed previously. The SH2 domain of SAP, the product of the mutated gene in human X-linked lymphoproliferative (XLP) disease, interacts with phosphorylated tyrosine-containing peptides as well as the corresponding non-phosphorylated sequences (Li *et al.*, 1999). This unusual recognition is achieved through contacts to the residues surrounding the Tyr residue in both the amino and carboxy direction (Poy *et al.*, 1999). As another example of atypical SH2 recognition, several SH2 domains have been shown to bind to phosphorylated serine or threonine containing motifs that lack a phosphorylated tyrosine residue (Cleghon &

Morrison, 1994; Dutartre *et al.*, 1998; Malek & Desiderio, 1994; Nantel *et al.*, 1998; Pendergast *et al.*, 1991). Additionally, there are SH2 mediated interactions that do not rely at all on the presence of a phosphorylated residue in the recognition motif. The Shc binding protein PAL (Protein expressed in Activated Lymphocytes) interacts specifically with the Shc SH2 domain and yet is not phosphorylated (Schmandt *et al.* 1999). As well, the interaction between the Lck SH2 domain and the 62-kDa cytosolic protein, p62, does not require phosphorylation of tyrosine, serine or threonine and is unaffected by mutation of the invariant arginine in the Lck SH2 domain (Joung *et al.*, 1996). It has been proposed that this interaction may make use of a distinct ligand binding surface on the SH2 domain.

SH3 domains are also capable of unconventional ligand recognition. An entirely proline-independent interaction has been characterized between the SH3 domain of the immune cell adaptor, SKAP55 and the novel peptide motif, RKXXYXXY (Kang *et al.*, 2000). This tyrosine-based motif appears to contact the SH3 domain surface in a manner that is similar, but not identical, to the proline-rich sequences previously studied. Another example of unusual SH3 binding selectivity has been demonstrated for Eps8, an SH3-containing substrate for receptor and non-receptor tyrosine kinases that has been implicated in mitogenic signaling (Fazioli *et al.*, 1993; Gallo *et al.*, 1997; Wong *et al.*, 1994). Identification of physiological ligands for Eps8 as well as screening of phage display peptide libraries has revealed a marked preference for a PXXDY sequence (Mongiovi *et al.*, 1999). The DY portion of this sequence is absolutely required for binding and the proline residue appears to be a specificity determining element. To date, the three-dimensional structures of

the SKAP55 and Eps8 SH3 domains bound to their respective non-PXXP containing ligands are not available to facilitate a comparison of the different modes of ligand recognition. Additionally, the aforementioned intramolecular SH3 ligand within the Src tyrosine kinase does not conform to the signature PXXP motif (Xu *et al.* 1997). The sequence, KPQTQGLA, contains a single proline residue and yet adopts the left-handed PPII helical conformation when bound in an intramolecular fashion to the SH3 domain of Src. The unconventional substrate selectivity exhibited by these SH2 and SH3 domains suggests that the molecular recognition events mediated by these small adaptor domains may be more diverse than previously believed.

SH2 domains themselves have been implicated as SH3 binding partners. The Crk-II SH2 domain contains a proline-rich insert in the DE loop that interacts with a subset of SH3 domains (Anafi *et al.*, 1996). This insert is not required for binding of the Crk-II SH2 domain to pTyr-containing sequences. However, pTyr ligand binding to the Crk-II SH2 domain does appear to enhance the interaction of the SH2 proline-rich insert with SH3 domains. Two other examples of SH3-SH2 interactions have been reported. Biochemical investigations have revealed intermolecular interactions between the SH2 and SH3 domains of the Src kinase family (Panchamoorthy *et al.*, 1994). These interactions appear to be specific since the Src family SH3 domains do not bind to the SH2 domains of non-Src family kinases such as Abl and PI3-K or to SH2 domains of the noncatalytic proteins Grb-2 and Crk. Additionally, the crystal structure of an SH2 and SH3 dual domain-containing fragment of Lck revealed a head-to-tail dimer where the SH3 domain of one monomer directly contacts the SH2 domain of the

neighboring monomer (Eck *et al.*, 1994). While this structure provides molecular details for the SH3-SH2 interaction which are lacking in the previously mentioned studies, the complex was not observed in solution and therefore questions can be raised as to the role of fortuitous crystal packing in stabilizing the interaction.

We have now characterized an *inter*molecular interaction *in solution* between the SH3 and SH2 domains of Itk. The interaction occurs between the singly expressed SH2 and SH3 domains of Itk as well as between two SH3/SH2 dual domain-containing fragments (Itk SH32). Using nuclear magnetic resonance (NMR) spectroscopy we have mapped out the residues of each domain involved in complex formation. The Itk SH3 binding pocket interacts in an intermolecular fashion with four loops (AB, CD, EF and BG, see figures 3&4) of the SH2 domain. In contrast to the Crk-II SH2 domain (Anafi *et al.*, 1996), the loops in the Itk SH2 domain do not contain a primary sequence that resembles the PXXP consensus motif for SH3 recognition. We have examined the ability of the Itk SH3 and SH2 domains to interact with the respective domains of non-Tec family kinases and have not detected binding. A comparison of the sequence homology between a representative group of SH2 domains provides a possible explanation for this observed specificity. Finally, we propose a functional relevance for this interaction in regulating Tec kinase activity.

## Results and Discussion

### Self-association of the Itk SH32 dual domain fragment

To investigate the possibility of self-association between the regulatory domains of Itk, a dual SH3 and SH2 domain-containing fragment of Itk (SH32) as well as the isolated Itk SH3 and SH2 domains were bacterially expressed and purified. $^{1}H$-$^{15}N$ heteronuclear single quantum correlation (HSQC) spectra of recombinant Itk SH32, Itk SH3 and Itk SH2 are shown in Figure 1 A, C & D. Chemical shift dispersion of the crosspeaks typical of folded protein is observed for each fragment of Itk, facilitating sequence specific resonance assignments. The crosspeaks in the single domain spectra (Fig. 1 C&D) are not exact subsets of the dual domain-containing fragment, Itk SH32. In addition to the overall line broadening observed, chemical shift perturbations as well as disappearance of some crosspeaks are observed for Itk SH32 as compared to the isolated domains. These spectral changes are either due to the covalent linkage between the two domains and/or arise from an interaction between domains. To test the possibility that the dual domain-containing fragment (Itk SH32) self-associates, a mutant of the Itk SH32 dual domain was constructed (Itk SH3*2(W208K)) in which Trp 208 (a critical residue in the SH3 binding pocket) was changed to a Lys. Comparison of the HSQC spectra for the single domains (Fig. 1 C&D) with the corresponding resonances of the Itk SH3*2(W208K) mutant (Fig. 1B) reveals only small chemical shift differences due to the mutated residue and similar linewidths. The observed line broadening taken together with changes in the resonance frequencies of a subset of residues suggests that Itk SH32 self-associates in a manner that requires an intact SH3 binding pocket.

**Association of the single domains and localization of the binding interface**

To simplify analysis of the residues that mediate the self-association of the Itk SH32 fragment in solution, we investigated the potential interaction of the isolated SH3 and SH2 domains. Titration of unlabeled Itk SH2 into an NMR sample of $^{15}$N-Itk SH3 results in significant perturbation of the chemical shift and/or change in linewidths of 30 of the 65 backbone $^{1}$H/$^{15}$N crosspeaks in the HSQC spectrum (Fig. 2A). Likewise, the reverse titration of unlabeled Itk SH3 into $^{15}$N-Itk SH2 causes changes in 33 of 104 backbone $^{1}$H/$^{15}$N crosspeaks in the HSQC spectrum (Fig. 2B). The observed shifts in both the SH3 and SH2 domains in these titration experiments correspond closely to the chemical shift perturbations observed in comparing Itk SH32 with the Itk SH3*2(W208K) mutant (Fig. 1A&B, Fig. 2). Thus, the contacts that mediate self-association between dual domain containing fragments of Itk also occur between the Itk SH3 and SH2 domains when expressed as separate fragments. The intermolecular association of the Itk SH3 and SH2 domains in solution has enabled us to use chemical shift mapping to investigate this interaction on a residue specific basis.

Complete sequence specific backbone resonance assignments for recombinant $^{15}$N/$^{13}$C doubly labeled Itk SH2 and SH3 domains have been carried out using standard triple resonance experiments. Identification of residues that shift upon addition of unlabeled Itk SH2 domain to $^{15}$N labeled Itk SH3 (Fig. 2A) reveal that the surface on the Itk SH3 domain involved in the interaction encompasses the well characterized ligand binding pocket formed by conserved aromatic residues. This is consistent with the observation that mutation of the conserved tryptophan (W208) in the SH3 binding pocket of the dual domain-containing

fragment abrogates self-association (Fig. 1B). Chemical shift perturbations in the labeled SH2 domain induced upon addition of unlabeled SH3 domain were used to map out the region of the SH2 domain involved in this interaction (Fig. 2B). The corresponding residues localize the Itk SH2 domain binding surface to four loops: AB, CD, EF and BG. These loops form a contiguous surface on the tertiary structure of other SH2 domains for which structures have been solved. The residues on the Itk SH2 domain involved in the interaction do not contain the conventional PXXP motif known to bind SH3 domains nor the PXXDY motif previously described as a binding site for the Eps8 SH3 domain. Thus, the Itk SH3-SH2 interaction has revealed a novel binding capacity of the Itk SH3 domain.

The fact that the Itk SH2 domain contacts the conserved ligand binding site of the SH3 domain suggests that the intermolecular SH3-SH2 interaction may play a role in regulating the intramolecular Itk proline-SH3 association described previously (Andreotti *et al.*, 1997). The juxtaposition and resulting intramolecular interaction of the proline-rich sequence and the SH3 receptor within Itk has been shown to limit the binding of other ligands to the SH3 binding pocket (Andreotti *et al.*, 1997). However, titration of unlabeled Itk Pro-SH3 into a sample containing $^{15}$N-Itk SH2 reveals chemical shift perturbations within the SH2 domain that are identical to those observed in the direct SH3-SH2 interaction (see Figure 2B). This result suggests that the intramolecular regulatory complex of Itk may be disrupted by intermolecular interactions between Itk molecules.

In the absence of a high-resolution structure of the Itk SH32 dual domain, we have used the previously determined structure of the Lck SH32 dual domain fragment (Eck *et al.*,

1994) to examine the nature of the binding surfaces mapped out for Itk by NMR. A surface map of the Lck SH32 dual domain fragment highlighting the residues that mediate the Itk SH3-SH2 interaction is shown in Fig. 3A. The chemical shift perturbations of the SH3 domain are localized to the conserved aromatic binding pocket along with residues that make up the more variable RT and n-Src loops. Unlike the SH3 domain, the chemical shift perturbations of the SH2 domain do not map out a previously characterized binding surface. The interaction between the Itk SH2 and SH3 domains involves four loop regions (AB, CD, EF, and BG) that link the regular secondary structural elements of the SH2 domain. The binding surface includes part of the well defined pTyr peptide binding site; the EF and BG loops form the ridges of the pY+3 pocket and contribute sidechains that determine the specificity of pTyr peptide binding. The binding interface extends to include the AB and CD loops forming a ridge along one face of the SH2 domain. There are a number of basic residues present in each of the four loop regions of Itk (see Figure 4) that may form critical electrostatic interactions with acidic residues present in the binding pocket of the Itk SH3 domain. For both the SH3 and SH2 domains, the chemical shift perturbations map out a relatively large region on the surface of the protein. This surface may be larger than the direct contact area of interaction because residues with binding-induced chemical shift perturbations generally define a region beyond the area involved in the direct protein-protein interaction (Spitzfaden *et al.*, 1992).

A comparison of the interaction surfaces in the dual SH32 domains of Itk and Lck (Eck *et al.*, 1994) reveals that both complexes exploit the conserved aromatic binding pocket

of the SH3 domain (Fig. 3B). In contrast, the SH2 residues involved in the Itk SH32 self-association are quite different from those that mediate Lck dimer formation. A small region on the EF loop of the SH2 domain is common to the interaction surfaces of the Lck dimer and the Itk complex. The remaining region on the SH2 domain that is involved in Lck dimer formation is primarily composed of three β-strands, βF, βE, and βD', that do not participate in the Itk self-association event. In addition, the region of the SH2 domain involved in Lck dimer formation is on a different 'face' of the molecule, suggesting a different relative orientation of the self-associating dual domain containing fragments of Lck and Itk.

**The Itk SH3-SH2 interaction is specific**

A sequence alignment of nine SH2 domains including that of Itk is shown in Fig. 4. The regions of the Itk SH2 domain involved in the interaction with the Itk SH3 domain are poorly conserved among the SH2 domains examined. Specifically, the primary amino acid sequences in loops CD, EF and BG in the Itk SH2 domain differ from the corresponding loop regions of other non-Tec family SH2 domains. This observation prompted us to test the ability of the Itk SH3 and SH2 domains to bind SH2 and SH3 domains from other proteins. Exploiting the fact that the interaction occurs between the individual domains, we performed HSQC titration experiments with SH3 and SH2 domains from different parent proteins. In contrast to the results for the equimolar mixture of Itk SH3 and Itk SH2 (Fig. 2A), significant chemical shift perturbations are *not* observed for the Itk SH3 domain in the presence of the Grb2 SH2 domain (data not shown). To test whether the Itk SH2 domain showed similar

specificity, we performed a titration experiment with the Lck SH3 domain and the Itk SH2 domain. Again, chemical shift perturbations were not observed. Therefore the SH3-SH2 interaction appears to be specific for the SH3 and SH2 domains of Itk, and this specificity may be related to the non-homologous nature of the amino acid sequence in the CD, EF and BG loops. The exact nature of the contacts that mediate the SH3-SH2 recognition event must await the availability of the three-dimensional structure of the Itk SH2 domain.

**Further biophysical characterization of Itk SH32**

As is evident in the previously determined structure of the Itk SH3 domain (Andreotti *et al.*, 1997), Trp 208 is centrally located and solvent exposed in the ligand binding pocket. For SH3 domains in general, this conserved Trp is indispensable for ligand binding; mutation removes the ability of the SH3 domain to mediate receptor-ligand interactions. We have demonstrated that the Itk SH3 binding pocket is involved in the interaction with the Itk SH2 domain and that mutation of Trp 208 prevents SH2 binding (Figure 1B). Thus, we anticipate that the sidechain of Trp208 may make direct contact with the Itk SH2 domain and that a comparison of NOE data for the SH3 domain alone and in the presence of the Itk SH2 domain may reveal interdomain NOEs.

Two-dimensional homonuclear NOESY spectra were acquired and crosspeaks from the well resolved Itk SH3 Trp208 indole NH were analyzed. For the Itk SH3 domain alone (Figure 5A), the NOEs observed are due to the intraresidue β protons and aromatic protons, δ1 and ζ2. In Figure 5B, the SH2 domain was added in an equimolar ratio to the SH3 domain,

and an identical NOESY experiment was carried out. In addition to the intraresidue NOEs observed for the SH3 domain alone, NOEs are observed from the Trp208 sidechain NH to resonances at 4.3 ppm and 1.3 ppm. These NOEs are not intraresidue NOEs (backbone assignments indicate that the Itk SH3 Trp208 α proton resonates at 5.1 ppm and a Trp sidechain will not have a proton resonance at 1.3 ppm) and may therefore be interdomain NOEs between the indole NH proton in the SH3 binding pocket and protons on the surface loops of the SH2 domain. An identical two-dimensional homonuclear NOESY experiment was carried out for the Itk SH32 dual domain fragment (Figure 5C). The same non-intraresidue NOEs from the Trp208 sidechain are observed (4.3 and 1.3 ppm) in addition to a weak upfield NOE at approximately 1.1 ppm. Filtered isotope experiments will be required to unambiguously identify the residues corresponding to these resonances and firmly establish that the observed NOEs are due to interdomain contacts. Nevertheless, given the solvent exposed nature of Trp 208 in the SH3 binding pocket, it is likely that the NOEs observed correspond to short proton-proton distances across the binding interface. Furthermore, the similarity in the NOE pattern for the Trp208 sidechain NH in both the 1:1 SH3/SH2 complex and the Itk SH32 dual domain (Fig. 5B&C), together with the agreement in the $^{1}H$ and $^{15}N$ resonance frequencies for each, suggests that the binding pocket of the Itk SH3 domain is occupied by the same region of the Itk SH2 domain in both cases.

The translational self-diffusion coefficient ($D_S$) of a molecular species can be determined from the attenuation of NMR signals in a field gradient echo experiment (Stejkal & Tanner, 1965), and has been shown to be a useful means of characterizing the self-

association of proteins under solution NMR conditions (Altieri *et al.*, 1995). NMR diffusion experiments were performed on 1.1 mM samples of Itk SH32 and Itk SH3*2(W208K) (Fig. 6). The difference between the curves shown in Fig. 6, though small, is clearly significant with respect to the estimated experimental error. A $\log(I/I_0)$ vs $(\Delta-\delta/3)(\gamma\delta G)^2$ plot will be linear for a homogenous, diffusing species, with the slope giving $D_S$. Inspection of Fig. 6 shows that the curve for Itk SH3*2(W208K) deviates only slightly from linearity, whereas the curve for Itk SH32 deviates quite significantly over the last nine data points. Such non-linearity is characteristic of molecular weight heterogeneity (von Meerwall, 1982; Haner & Schleich, 1989; Price *et al.*, 1999). In such a case, the initial and final slope of the curve give maximum and minimum values of the distribution of $D_S$, and a fit to the entire curve provides an average value of the diffusion coefficient. Average values of $D_S$ were obtained for both samples by fitting each complete data set to Eq. (1) via non-linear regression (see Table 1 and Materials & Methods). The WT data did not fit well to Eq.(1). Consequently, the estimated error for $D_S(WT)_{ave}$ is relatively large. Therefore, the last nine points of the WT data were fit independently to estimate the limiting minimum value $D_S(WT)_{min}$ which would correspond to the largest molecular weight species in the solution.

The data indicate that Itk SH32 is involved in oligomerization, probably a monomer-dimer equilibrium, whereas the Itk SH3*2(W208K) mutant remains monomeric. The expected ratio of diffusion coefficients for a monomer-dimer system, based upon a simple hard-sphere hydrodynamic model for frictional coefficients is $D_S(Dimer)/D_S(Monomer) = 0.75$ (Teller *et al.*, 1979). Our observed ratio is somewhat higher: $D_S(WT)_{min}/D_S(W208K)_{ave}$

= 0.79. It is overly simplistic to model the Itk SH32 dual domain as a hard sphere. If we regard the dual domain monomer itself as a string of two hard spheres (still a very crude model) we obtain from the hard-sphere hydrodynamic model $D_S$(Dimer)/$D_S$(Monomer) = 0.77 for a tetrahedral packing arrangement, and $D_S$(Dimer)/$D_S$(Monomer) = 0.81 for a square packing arrangement. Both cases are in reasonable agreement with the observed ratio. These results are consistent with the uniformly larger linewidths observed in the HSQC spectrum of Itk SH32 relative to the corresponding linewidths in the HSQC spectrum of the Itk SH3*2(W208K) mutant (see Fig. 1A&B). Additionally, intramolecular contacts between the SH3 and SH2 domains within a single dual domain fragment are unlikely due to steric limitations (Nam *et al.*, 1996; Gosser *et al.*, 1995; Maignan *et al.*, 1995; Eck *et al.*, 1994; Xu *et al.*, 1999; Fushman *et al.*, 1999).

The presence of an Itk SH32 dimer in solution was also detected using analytical ultracentrifugation. Equilibrium sedimentation was performed on Itk SH32 and the Itk SH3*2(W208K) mutant to determine that the Itk SH32 self-association occurs at concentrations lower than that used for NMR and to estimate the dissociation constant ($K_d$). Sedimentation behavior was evaluated at three different concentrations: 26.6 μM, 13.3 μM, and 6.65 μM for both Itk SH32 and Itk SH3*2(W208K). With increasing concentration the apparent molecular mass for Itk SH32 increased, whereas that for Itk SH3*2(W208K) remained constant. This verifies the existence of a monomer-dimer equilibrium for Itk SH32 as determined by NMR, and confirms that the Itk SH3*2(W208K) mutant does not self-associate. Using the equilibrium sedimentation data, we have determined that the dissociation

constant ($K_d$) for dimerization of Itk SH32 is approximately 25 μM. The measured $K_d$ is consistent with the exchange regime observed in the NMR data (intermediate to fast), which gives a $K_d \geq 10^{-7}$ M, assuming diffusion-limited molecular association.

**Ligand Binding Studies**

Inspection of the SH3 and SH2 domain surfaces responsible for mediating the SH3-SH2 interaction suggests that conventional SH3 and SH2 ligands may play a role in regulating the intermolecular interaction of the Itk SH32 dual domain. The conserved aromatic SH3 binding pocket is used for both polyproline recognition and SH2 binding which suggests that the two binding events may be mutually exclusive. In contrast, comparison of the region of the SH2 domain that contacts the SH3 domain with that involved in phosphopeptide recognition reveals a much smaller shared surface around the pY+3 binding pocket (Figure 3A). Thus, given the overlap in the binding sites on both the SH3 and SH2 domains, we compared peptide ligand binding to the isolated domains and to the self-associating dual domain.

The proline-rich peptide (QQPPVPPQRPMA) and the phosphotyrosine-containing sequence (ADpYEPPPSNDE) were synthesized by standard solid phase methods and purified to homogeneity. Both peptide sequences are derived from Slp-76, a phosphoprotein that binds to full-length Itk via interactions with both the SH3 and SH2 domains (Bunnell *et al.*, 1996; Bunnell *et al.*, 2000; Su *et al.*, 1999). Four separate HSQC titrations were carried out to determine dissociation constants for each peptide bound to its corresponding target

domain in isolation and in the context of the self-associating SH32 dual domain. Based on chemical shift perturbation assays where the peptide/protein ratio varied between 0 and 3, a $K_d$ of 0.77 ± 0.16 mM for the isolated SH3-polyproline peptide interaction and a $K_d$ of 0.41 ± 0.12 mM for the isolated SH2-pTyr peptide interaction were determined. The affinities of these physiological peptides are less than the binding affinities observed for peptides selected from a library screening yet are consistent with competition experiments reported previously for the same peptides binding to the domains of Itk (Bunnell *et al.* 2000). The corresponding determination of peptide binding affinities to the Itk SH32 dual domain is complicated by concomitant dimer dissociation.

For binding of the pTyr-containing sequence to the SH2 domain of Itk SH32, we were able to monitor chemical shift changes for residues that make up the peptide binding site but do not participate in stabilizing the dimer interface. The chemical shift values of this subset of residues are unaffected by dimer dissociation yet are perturbed upon addition of ligand allowing for an estimate of the $K_d$ for pTyr peptide binding to the dual domain. Binding curves for binding of the pTyr peptide to both the isolated Itk SH2 domain and the Itk SH32 dual domain are shown in Figure 7. The difference in the curves reveals a lower binding affinity for interaction of the pTyr-peptide with the self-associating dual domain as compared to the single SH2 domain. This result is consistent with our model for homodimerization of Itk SH32 where part of the peptide binding site on the SH2 domain contacts the SH3 domain in an intermolecular fashion (Figure 3A) and therefore reduces peptide binding affinity. This result is in contrast to previous studies (Gosser *et al.*, 1995)

where peptide binding to isolated SH2 and SH3 domains was indistinguishable from binding to an SH32 dual domain.

Overlap in the binding sites for the SH2 domain and polyproline peptide ligand on the Itk SH3 domain made estimation of a dissociation constant unreliable. Each residue involved in peptide binding also experienced chemical shift perturbation as a result of dimer dissociation and was consequently not a faithful representation of peptide association. We therefore assessed the degree to which peptide association shifted the equilibrium toward the monomeric form of the Itk dual domain, *i.e.*, toward the Itk SH3*2(W208K) chemical shifts. A comparison of the resonance frequency of a given residue in an HSQC spectrum of the ItkSH3*2(W208K) mutant, Itk SH32, and Itk SH32 in the presence of excess polyproline peptide (Figure 8A) reveals a shift toward monomer when peptide is present. This suggests that the Slp-76 proline-rich peptide partially displaces the SH2 domain from the SH3 binding pocket at the concentrations tested. In contrast, a similar analysis for pTyr-peptide binding reveals very little if any shift toward the monomeric state (Figure 8B). This suggests that phosphopeptide does not dissociate the dimer to a significant extent. The extent to which the pTyr-containing peptide binds to Itk SH32 (Figure 7) may be due to contacts between the pTyr residue and the pTyr binding pocket on the SH2 domain which lies outside of the SH3 binding surface.

## Conclusions

Self-association of the SH3 and SH2 dual domain-containing fragment of Itk has been examined. Chemical shift perturbation data indicate a contiguous binding surface that differs from the Lck dimer interface previously observed. We have also shown on the basis of NMR diffusion and linewidth data that Itk SH32 forms a homodimer under NMR solution conditions. A point mutation that abrogates the interaction also completely eliminates dimerization. The region of the SH3 domain involved in the interaction encompasses the conserved ligand binding pocket that generally contacts polyproline sequences in the SH3-mediated interactions studied to date. The interaction surface on the SH2 domain extends along a ridge formed by residues in four loop regions. Notably, the amino acid sequences of the SH2 loops that contact the SH3 binding site do not resemble the standard PXXP motif that is regarded as the canonical SH3 ligand. Furthermore, the SH3 ligand within this SH2 domain does not arise from a contiguous amino acid sequence that is the hallmark of the prototypical SH3 ligand. It is conceivable that the loops on the SH2 domain adopt a spatial arrangement that can mimic the SH3-PXXP interaction. A detailed understanding of this molecular recognition will require further structural analysis.

The SH2 residues involved in the interaction partially overlap those responsible for phosphotyrosine peptide binding and the SH3 residues that mediate polyproline peptide binding and SH2 recognition are nearly identical. We have demonstrated that the binding affinity of a pTyr-containing peptide for the Itk SH2 domain diminishes in the presence of self-association and that excess peptide ligand for either the SH3 or SH2 domain does not

completely abrogate self-association. Given the fact that the binding assays were done using short peptide models, it is conceivable that the Itk/Slp-76 interaction within the cell may effectively compete with Itk self-association. Certainly variations in local concentration as a result of co-localization of two signaling proteins could modulate affinity to favor either self-association or interaction with exogenous protein.

The mechanism of Tec family kinase activation following receptor stimulation appears to follow a series of steps that include membrane redistribution and phosphorylation of key tyrosine residues. Stimulation causes an increase in levels of phosphatidylinositol 3,4,5-trisphosphate which is a high affinity ligand for the PH domain of the Tec kinases (Carpenter & Cantley, 1996; Rameh *et al.*, 1997). Studies of Btk regulation have suggested that the PH domain-phosphatidylinositol interaction is responsible for localizing Btk to the plasma membrane where activation proceeds (Scharenberg *et al.*, 1998; Kawakami *et al.*, 1994; Li *et al.*, 1997; Bolland *et al.*, 1998). Whether Itk is recruited to the membrane in the same fashion or is constitutively present at the membrane while other signaling partners are co-localized remains uncertain (Shan & Wange, 1999). In any event, an intact PH domain (capable of membrane association) appears to be a prerequisite for Tec family kinase function (Hyvönen & Saraste, 1997). Once membrane localized, the Tec kinases are phosphorylated by co-localized, activated Src kinases (Rawlings *et al.*, 1996) on a tyrosine residue in the kinase domain (Y551 in Btk). This transphosphorylation event occurs rapidly following stimulation and is followed by a slower autophosphorylation event on a conserved tyrosine residue in the ligand-binding pocket of the SH3 domain (Y223 in Btk) (Nisitani *et al.*, 1999).

This SH3 autophosphorylation step appears to be required for full kinase activation. Our current model of the conformational state of inactive Itk has the SH3 binding-pocket occupied by the proline-rich region of the adjacent TH domain in an intramolecular fashion (Andreotti *et al.*, 1997). A mechanism for displacement of the intramolecular proline ligand seems necessary for autophosphorylation to occur.

Based on the SH3-SH2 interaction we have described, we envision that an intermolecular association between the SH2 domain of one membrane bound protein and the SH3 domain of an adjacent molecule might effectively displace the intramolecular proline sequence from the SH3 binding pocket to promote kinase activation. The Itk Pro-SH3 fragment, in which the SH3 pocket is occupied by the proline-rich stretch (Andreotti *et al.*, 1997), binds to the Itk SH2 domain in a manner that is indistinguishable from that of the SH3-SH2 interaction described. Thus, it is conceivable that the SH3-SH2 interaction could compete with the intramolecular proline sequence for the binding pocket of the SH3 domain. Removal of the intramolecular proline sequence by a *transient* intermolecular SH3-SH2 interaction could accomplish the following: accessibility to the SH3 binding pocket for either association of exogenous ligand (high local concentration may compete with homodimer) or tyrosine autophosphorylation. As well, the SH3-SH2 interaction would reveal the internal proline sequence for association with other cellular ligands in the receptor mediated signaling pathway. The relatively weak affinity (~25 μM) observed for the self-association of Itk SH32 is entirely consistent with such a regulatory role. The effective affinity of the intermolecular interaction, which is related to the extent of homodimerization, could be

controlled by changes in the local concentration of Itk at the membrane during different signaling events.

Homodimerization has previously been observed for a fragment of another Tec kinase family member. The PH domain of Btk crystallizes as a dimer in the asymmetric unit (Hyvönen & Saraste, 1997) and it has been proposed that dimerization of PH domains on the membrane could be an important step in the activation of Btk (Baraldi *et al.*, 1999). It is conceivable that additional regulatory domains outside of the PH domain may be involved in stabilizing an oligomeric state at the membrane that ultimately results in active kinase.

Btk has garnered tremendous interest due to the fact that mutations in the gene coding for this protein cause the severe hereditary immunodeficiency, X-linked agammaglobulinemia (XLA). The disease is characterized by extremely low immunoglobulin levels due to a B cell differentiation defect (Mattsson *et al.*, 1996; Smith *et al.*, 1994). DNA mutations causing XLA occur in all five domains of Btk (PH, TH, SH3, SH2 & kinase) which implies that all five domains of the protein are required for proper regulation and signaling (Vihinen *et al.* & Smith, 1998). Nine missense mutations have been mapped to the SH2 domain of Btk and the positions of these point mutations are indicated on the Itk sequence (Figure 4). Also highlighted in Figure 4 are those SH2 residues for which the amide resonance frequencies change upon addition of the Itk SH3 domain. There is a striking correlation between the Btk SH2 XLA causing mutations and the loops of the Itk SH2 domain that we have determined mediate the observed SH2-SH3 interaction. Six of the nine SH2 loss of function mutations occur at positions directly involved in the SH3-SH2 interaction (as determined by chemical

shift mapping) while two missense mutations are immediately adjacent to an interacting residue. It has been previously suggested that a subset of the XLA causing mutations may disrupt the ability of the SH2 domain to bind phosphotyrosine-containing substrates (Mattsson *et al.*, 2000; Vihinen *et al.* & Smith, 1998). We would suggest that the XLA causing mutations may also abrogate the SH3-SH2 interaction described here. Certainly, the striking correlation between the surface loops of the Itk SH2 domain that we have determined mediate an interaction with the Itk SH3 domain and the XLA causing mutations identified to date suggests that the SH3-SH2 interaction may play an important role in the activation and subsequent signaling of Tec family kinases. The interaction we have described here has revealed a novel binding capability of the ubiquitous SH3 and SH2 domains and has identified a new interaction within the Tec kinase family that may modulate aspects of hematopoetic cell signaling.

## Materials and Methods

### Protein expression and purification

Inserts encoding the desired domains of Itk were generated by PCR and were subcloned into the BamHI site of pGex-2T to generate glutathione-*S*-transferase (GST) fusion proteins. The SH3 and SH32 5' primers encode Itk products beginning at amino acid 171, and the SH2 5' primer begins at amino acid 231. The SH3*2(W208K) mutant was constructed as described previously (Andreotti *et al.*, 1997). All fusion proteins were produced in *E. coli* strain BL21(DE3), and grown at 37°C in LB medium or modified M9 minimal medium; 50

μg/ml ampicillin. The modified M9 minimal medium used for the preparation of uniformly $^{15}N/^{13}C$ double labeled or $^{15}N$ single labeled protein contained $^{15}N$-enriched ammonium chloride (1g/L, Cambridge Isotope Laboratories) and either $^{13}C$-enriched glucose (2g/L, Cambridge Isotope Laboratories) or unenriched glucose as sole nitrogen and carbon sources. When the optical density (600 nm) reached 0.6, protein expression was induced by addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and the temperature was reduced to 30°C. Seven hours post-induction in LB medium or 17 hours post-induction in M9 minimal medium the cells were collected by centrifugation, resuspended in 50mM potassium phosphate buffer, pH 7.4, 75 mM NaCl, and 2 mM DTT, lysozyme was added, and the cells were stored at -80°C for 12 hours. Cells were lysed upon thawing at room temperature with addition of protease inhibitor (1mM PMSF) and DNAse (50 ul of 10 mg/ml stock). The cell lysate was clarified by centrifugation, and the resulting supernatant was purified using a glutathione-agarose column (Sigma). The fusion proteins were eluted with 5 mM glutathione, 50 mM potassium phosphate buffer, pH 7.4, 75 mM NaCl, 2 mM DTT, and 0.02 % $NaN_3$. Proteins were concentrated and loaded onto a gel filtration column (Sephacryl S-100 HR, Amersham Pharmacia Biotech). Fractions containing the fusion proteins were combined, and the concentration of NaCl was increased to 150 mM for thrombin cleavage to remove the GST tag. The proteins were placed at room temperature for approximately 1-2 hours (Itk SH2) or 12 hours (Itk SH3, SH3SH2) for complete cleavage. After cleavage, the proteins were passed over a glutathione column to remove GST, concentrated and loaded onto the gel filtration column. Fractions of protein were collected, analyzed for purity by SDS-

polyacrylamide (15%) gel electrophoresis and concentrated to 1-2 mM for NMR. All samples were stored at 5° C, except when NMR spectra were acquired.

**NMR spectroscopy**

NMR spectra were acquired using a Bruker DRX500 spectrometer operating at $^{1}H$ frequency of 499.867 MHz. A 5 mm triple resonance ($^{1}H/^{13}C/^{15}N$) probe with XYZ field gradients was used for all experiments. All spectra were collected at 298 K unless otherwise indicated. A gradient-enhanced HSQC experiment with minimal water saturation (Mori *et al.*, 1995) was used for all $^{1}H$-$^{15}N$ correlation experiments. 3D $^{15}N$-edited TOCSY and $^{15}N$-edited NOESY (Talluri & Wagner, 1996) spectra were acquired for a $^{15}N$ labeled Itk SH2 sample using mixing times of 100 ms and 72 ms, respectively. 3D CBCA(CO)NH, HNCACB (Muhandiran & Kay, 1994), and HCCH-TOCSY (Kay *et al.*, 1993) spectra were acquired for an $^{15}N/^{13}C$ doubly labeled sample of Itk SH2 using standard experimental protocols. The 3D data were used to obtain sequential assignments of the backbone $^{1}H$ and $^{15}N$ shifts of the SH2 domain. Sequential assignments for the Itk SH3 domain were completed previously (Andreotti *et al.*, 1997). Additional homonuclear 2D NOESY spectra were collected of Itk SH3, an equimolar SH3/SH2 mixture and the SH3/SH2 dual domain (Itk SH32), to identify NOEs involving W208 of SH3. For these spectra a mixing time of 175 ms and a sample temperature of 301 K were used. Data were processed by use of NMRPipe (Delaglio *et al.*, 1995) software, and the NMRView (Johnson & Blevins, 1994) software package was used to facilitate the assignment process.

**NMR Diffusion Measurements**

The self-diffusion coefficient $D_S$ was measured at 298 K for wild type and mutant dual domain protein samples by use of a gradient longitudinal encode-decode (LED) experiment (Tanner, 1970; Altieri *et al.*, 1995). The standard LED pulse sequence was modified by the use of bipolar encode-decode gradient pulses (Wider *et al.*, 1994) and by the addition of a WATERGATE module (Piotto *et al.* 1992). For each sample, a series of 1D NMR spectra was acquired during which the strength of the rectangular encode-decode gradient pulses was incremented from 0.6 to 35 G/cm. The NMR signal amplitude of a homogenous diffusing species is attenuated according to the Stejkal-Tanner relation (Stejkal & Tanner, 1965)

$$I = I_0 \exp[-(\gamma\delta G)^2 (\Delta - \delta/3) D_S] \qquad (1),$$

where G is the encode-decode gradient strength, I is the observed NMR signal intensity, $I_0$ is the signal intensity for G = 0, $\gamma$ is the $^1H$ gyromagnetic ratio, $\delta$ is the gradient duration, $\Delta$ is the time between encode and decode pulses and $D_S$ is the self-diffusion coefficient. Each series of 1D experiments was repeated four times in order to estimate the experimental error. A portion of the spectral envelope, consisting of the methyl proton region, was integrated to obtain the signal intensity. The z-axis gradient coil of a Bruker 5 mm TXI triple axis gradient probe head was used to generate the gradient pulses. The spectrometer temperature controller was calibrated using a standard methanol sample (van Geet, 1970). The gradient strength G was calibrated by measuring the decay of residual HOD in 99.98% $D_2O$, using the

literature value of $D_S$ = 1.902 × $10^{-5}$ $cm^2$/sec (Longsworth, 1960), and fitting the data to Eq. (1) by non-linear regression. The procedure was tested by measuring $D_S$ for 15 mM lysozyme, giving a value of 1.07 × $10^{-6}$ $cm^2$/sec (data not shown), in good agreement with the reported value of 1.08 × $10^{-6}$ $cm^2$/sec (Altieri *et al.*, 1995).

**Analytical Equilibrium Ultracentrifugation**

Analytical ultracentrifugation experiments were performed on a Beckman Optima XL-A ultracentrifuge. Itk SH32 and Itk SH3*2(W208K) samples were prepared as described for the NMR samples; however, concentrations of 6.65 μM, 13.3 μM, and 26.6 μM were used. Protein samples were centrifuged at least 12 hours before data collection. Samples at each concentration were sedimented at rotor speeds of 22,000, 26,000, and 30,000 rpm, and the temperature was maintained at 25°C. Stepwise radial scans were performed at 280 nm and each reading was an average of 30 points with nominal spacing of 0.001 cm between radial positions. The absorbance readings were measured at 1 hour intervals to ensure that equilibrium had been reached. Three scans for each rotor speed were averaged, and the data were used to calculate the apparent molecular weight ($M_w$) for each concentration. The $M_w$ is a measure of the average molecular weight of the monomer-dimer solution at equilibrium for the different concentrations:

$$M_W = f_M M_M + (1 - f_M) M_D \qquad (2)$$

Where $f_M$ and (1-$f_M$) are the weight fractions of monomer and dimer respectively, and $M_M$ and $M_D$ are the molecular weights of monomer and dimer. The data was plotted as molecular

weight versus concentration and a curve was obtained by a nonlinear least squares fit to Equation 2. Equation 3 can be substituted for $f_M$ in Equation 2 and the equilibrium constant ($K_d$) was extracted from the fitted curve.

$$f_M = \frac{-K_d + \sqrt{K_d^2 + 8K_d P_T}}{4P_T} \qquad (3),$$

where $P_T$ is the total protein concentration and $K_d$ is the equilibrium dissociation constant. The curve was constrained to the monomer molecular weight obtained for Itk SH3*2(W208K) from ultracentrifugation data (14.8 kDa). This weight is lower than the calculated molecular weight of 19.7 kDa. Both the Itk SH32 and Itk SH3*2(W208K) samples were analyzed by NMR prior to ultracentrifugation experiments and the number of peaks in the HSQC spectra agreed with the corresponding amino acid sequences. Therefore, Itk SH32 and ItkSH3*2(W208K) do not exhibit ideal hydrodynamic behavior during ultracentrifugation.

**NMR Binding Assays**

Solutions of $^{15}$N-labeled Itk SH2, SH3 and SH32 dual domain were prepared in 400 μL of phosphate buffer as described above. The solutions were 0.5 mM for the single domain proteins and 1.0 mM for the dual domain protein, as determined by UV-visible absorption at 280 nm. Stock solutions of each peptide were prepared in 20 μL $H_2O$. The peptide solutions were prepared (as determined by the dry weight of lyophilized peptide) to reach a threefold molar excess of peptide at the end of each titration, neglecting the small

volume of added peptide. For each protein sample, 2 μL aliquots of peptide solution were added so that the molar ratio of peptide to protein was increased from 0 to 3 in 11 steps. After each addition, an $^{15}N$-$^{1}H$ HSQC spectrum was acquired.

The NMR spectra were examined and peaks were selected that displayed significant chemical shift perturbation and were well resolved in both single and dual domain spectra over the course of the titrations. It should be noted that for the Itk SH32-pTyr peptide titration, peaks corresponding to the peptide binding region that lay outside of the dimer interface were used to generate binding curves. The chemical shift perturbations in both the $^{1}H$ and $^{15}N$ dimensions were used to obtain binding curves. The chemical shift of protein/ligand system in fast exchange is described by Equation 4 (Lian & Roberts, 1993):

$$\delta_{obs} = \delta_P + (\delta_{PL} - \delta_P)\frac{L_T + P_T + K_d - \sqrt{(L_T + P_T + K_d)^2 + 4P_T}}{2P_T} \quad (4),$$

where $\delta_{obs}$ is the observed protein chemical shift, $\delta_P$ is the protein chemical shift in the absence of ligand, $\delta_{PL}$ is the protein chemical shift in the presence of excess ligand, $L_T$ is the total ligand concentration, $P_T$ is the total protein concentration and $K_d$ is the equilibrium dissociation constant. Figure 7 shows representative plots of $\delta_{ob}$ vs. $L_T$. The parameters $K_d$ and $\delta_{PL}$ were determined by fitting the data to Equation 4 by non-linear regression. For each protein/ligand combination (except SH32 dual domain and polyproline ligand) at least three independent $K_d$ values were determined from which average values and estimated experimental errors (three standard deviations) were calculated.

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Table 1.

Self-diffusion coefficients of 1.5 mM Itk SH32 and Itk SH3*2(W208K) at 298 K

| Protein | $D_S$ ( $\times 10^{-7}$ $cm^2/sec$) | $D_S/D_S(W208K)_{ave}$ |
|---|---|---|
| $(W208K)_{ave}$ | 8.59 ± 0.09 | 1.00 |
| $(WT)_{ave}$ | 8.35 ± 0.21 | 0.97 |
| $(WT)_{min}$ | 6.81 ± 0.07 | 0.79 |

**Figure 1.** $^{1}H$-$^{15}N$ HSQC spectra of purified, recombinant (A) Itk SH32, (B) Itk SH3*2(W208K), (C) Itk SH3 and (D) Itk SH2. For each sample protein concentration was between 1 and 1.3 mM in 50 mM potassium phosphate, pH 7.4, 75 mM NaCl, 2 mM DTT, 0.02% $NaN_3$, 25 $^{0}C$. $^{1}H$ and $^{15}N$ chemical shifts are referenced to external DSS at 0 ppm.

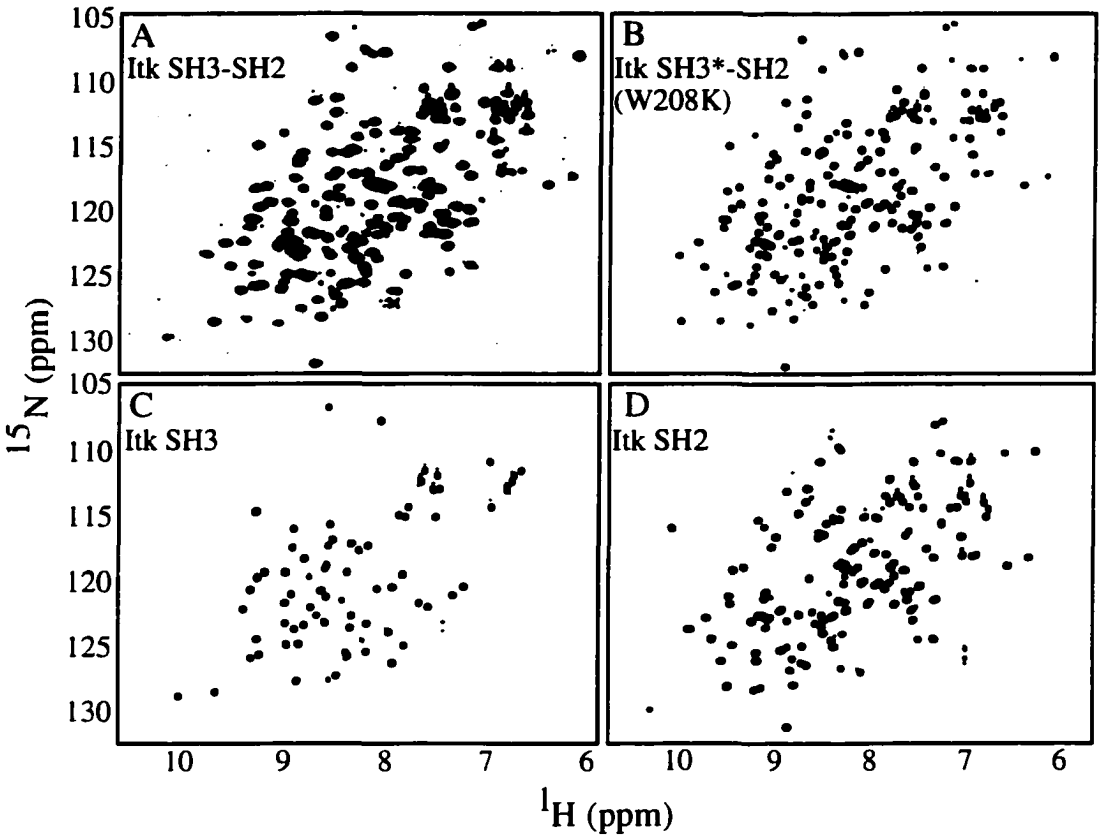

A
Itk SH3-SH2
B
Itk SH3*-SH2
(W208K)
C
Itk SH3
D
Itk SH2
105
110
115
120
125
130
10
9
8
7
6
$^{15}$N (ppm)
$^{1}$H (ppm)

**Figure 2.** Chemical shift changes in the isolated $^{15}$N-labeled SH3 (A) and SH2 (B) domains of Itk induced upon addition of either the unlabeled Itk SH2 or SH3 domain, respectively. *Left panels,* Selected expanded regions of $^{1}$H-$^{15}$N HSQC spectra (blue) of Itk $^{15}$N-SH3 (A) or Itk $^{15}$N-SH2 (B) superimposed on $^{1}$H-$^{15}$N HSQC spectra (red) of the 1:1 complexes of labeled Itk $^{15}$N-SH3 and unlabeled Itk SH2 (A) or Itk $^{15}$N-SH2 and unlabeled Itk SH3 (B). Arrows show the direction of the observed shifts. All residues with shifts greater than 0.1 ppm in either the proton or nitrogen dimension were considered significant. The titration of the unlabeled Itk Pro-SH3 fragment and Itk $^{15}$N-SH2 results in identical shifts to those observed in (B). *Right panels,* Residue-by-residue differences (thick bars) in $^{15}$N (top) and $^{1}$HN (bottom) Itk SH3 (A) and SH2 (B) chemical shifts. The plotted values correspond to chemical shift changes observed in the dual domain Itk SH32 as compared to the mutant Itk SH3*2(W208K). $\Delta\delta = (\delta_{free} - \delta_{bound})$ where $\delta_{free}$ refers to resonances in Itk SH3*2(W208K) and $\delta_{bound}$ refers to the chemical shift values of $^{15}$N-labeled dual domain Itk SH32. The thin lines indicate residues for which line broadening occurred to an extent that the HSQC signal disappears. For 2A, the last two columns are the chemical shift perturbations of the W208 and W209 side chain indole $^{1}$HN and $^{15}$N resonances. (For W208, $\delta_{free}$ for the singly expressed SH3 domain was used to calculate $\Delta\delta$.)

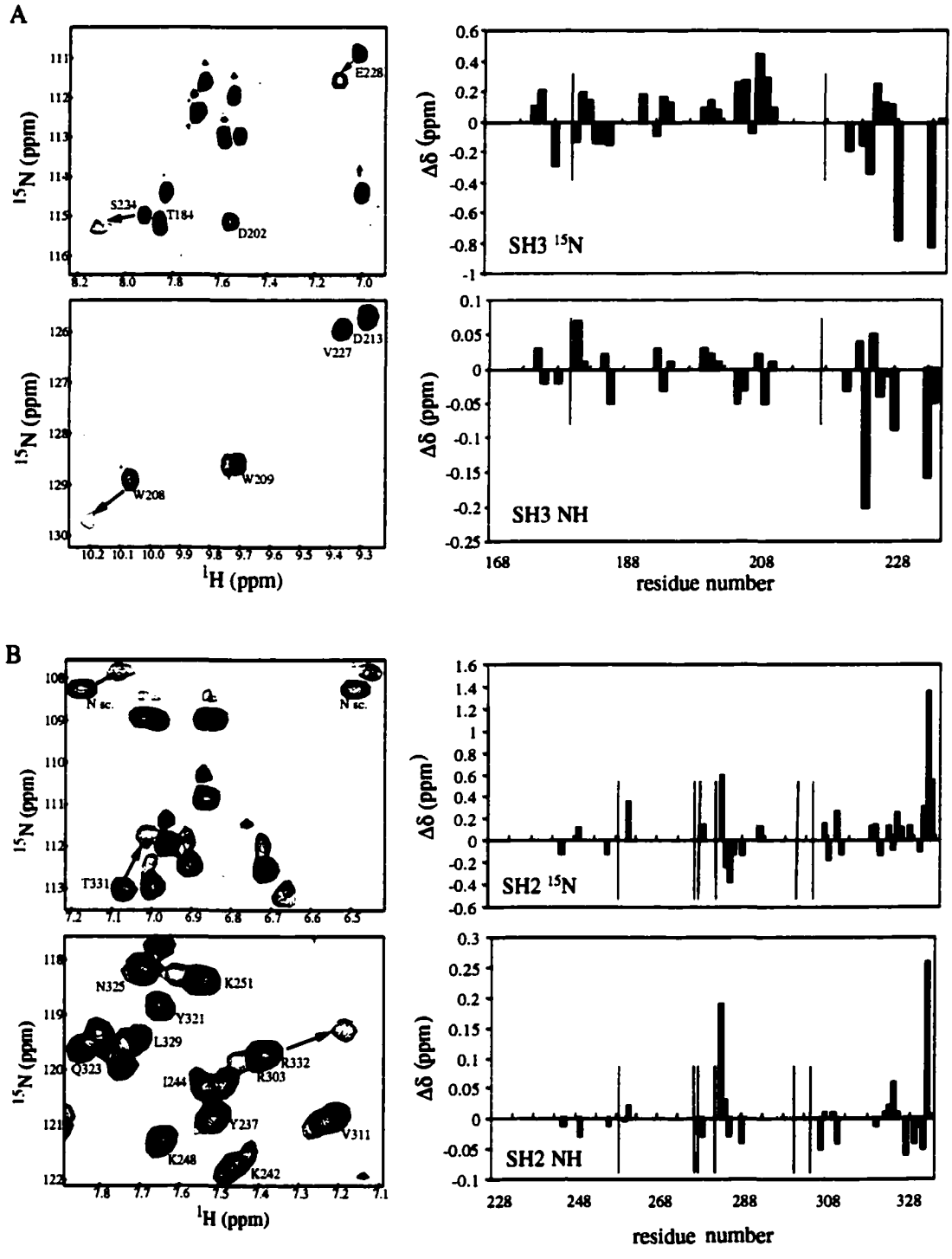

A
$^{15}$N (ppm)
E228
S234
T184
D202
D213
V227
W209
W208
$^{1}$H (ppm)
Δδ (ppm)
SH3 $^{15}$N
SH3 NH
residue number
B
N sc.
T331
N325
K251
Y321
L329
Q323
R332
R303
I244
Y237
V311
K248
K242
SH2 $^{15}$N
SH2 NH

**Figure 3.** Surface maps of the Lck SH32 dual domain fragment (Eck *et al.*, 1994) (A) The residues involved in the Itk SH32 self-association as determined by chemical shift perturbations (arrived at by analysis of the isolated domain titration experiments as well as comparison of the HSQC spectra for wild type Itk SH32 and mutant Itk SH3*2) are indicated in red while residues that do not show any change in their resonance frequencies upon addition of binding partner are in blue. Labels in the SH3 domain include Lck Trp 97, which corresponds to Itk Trp 208, and the variable n-Src and RT loops that line the conserved aromatic binding pocket. The phosphotyrosine peptide binding site on the SH2 domain is indicated by labeling of the phosphotyrosine (pY) and carboxy-terminal (pY+3) pockets. The EF, BG, AB and CD loops are labeled accordingly. (B) Comparison of the surfaces mediating the SH3-SH2 interaction in the Lck dual domain crystal structure (yellow) (Eck *et al.*, 1994) and the Itk SH3-SH2 interaction (red, as in A). Orange indicates an overlap in the regions involved in each interaction. The surface maps were calculated using Molecular Analysis and Molecular Display (MolMol) (Koradi *et al.*, 1996) from the PDB file 1LCK.

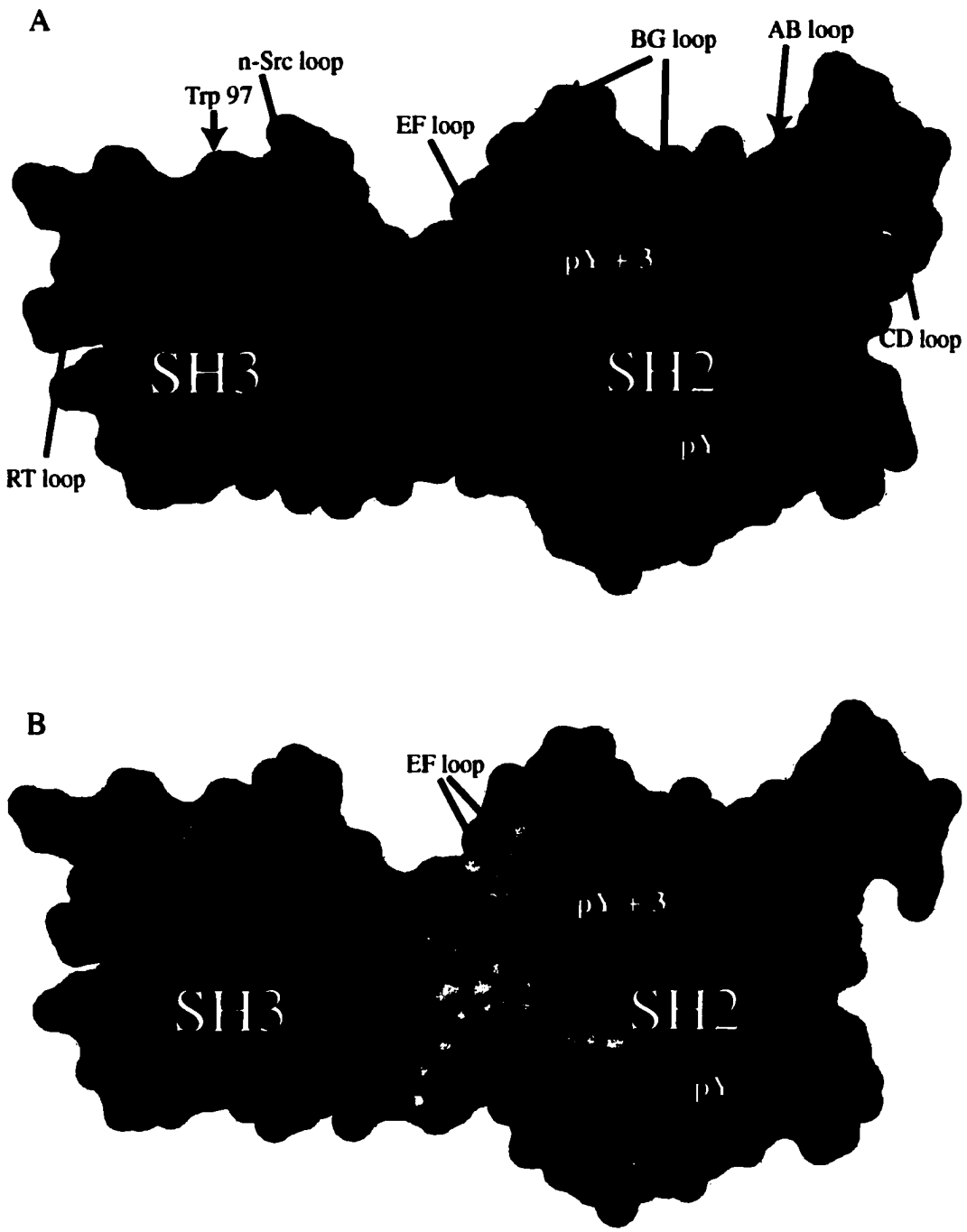

A
n-Src loop
Trp 97
EF loop
BG loop
AB loop
pY + 3
CD loop
SH3
SH2
pY
RT loop
B
EF loop
pY + 3
SH3
SH2
pY

**Figure 4.** Aligned sequences of the SH2 domains from nine different proteins. The Tec family SH2 domains (Itk and Btk) are on the first two lines. Numbering refers to full-length Itk. The location of regular secondary structure is based on the Lck sequence (Eck *et al.*, 1994). Residues conserved among SH2 domains are in blue and the portions of the Itk sequence highlighted in red are the SH2 residues that are involved in the interaction with the Itk SH3 domain as determined by chemical shift perturbations. Asterisks indicate the position of the Btk missense mutations that have been identified to date in patients with XLA. Underlined residues mediate pTyr peptide binding in other SH2 domains for which complex structures have been solved. Accession numbers for each sequence are as follows: Itk: Q03526, Btk: P35991, Blk: NP_031575, Src: TVCHS, Fyn: CAB10788, Lck: P06239, Abl: AAB60393, Grb2: NP002077, Csk: NP004374.

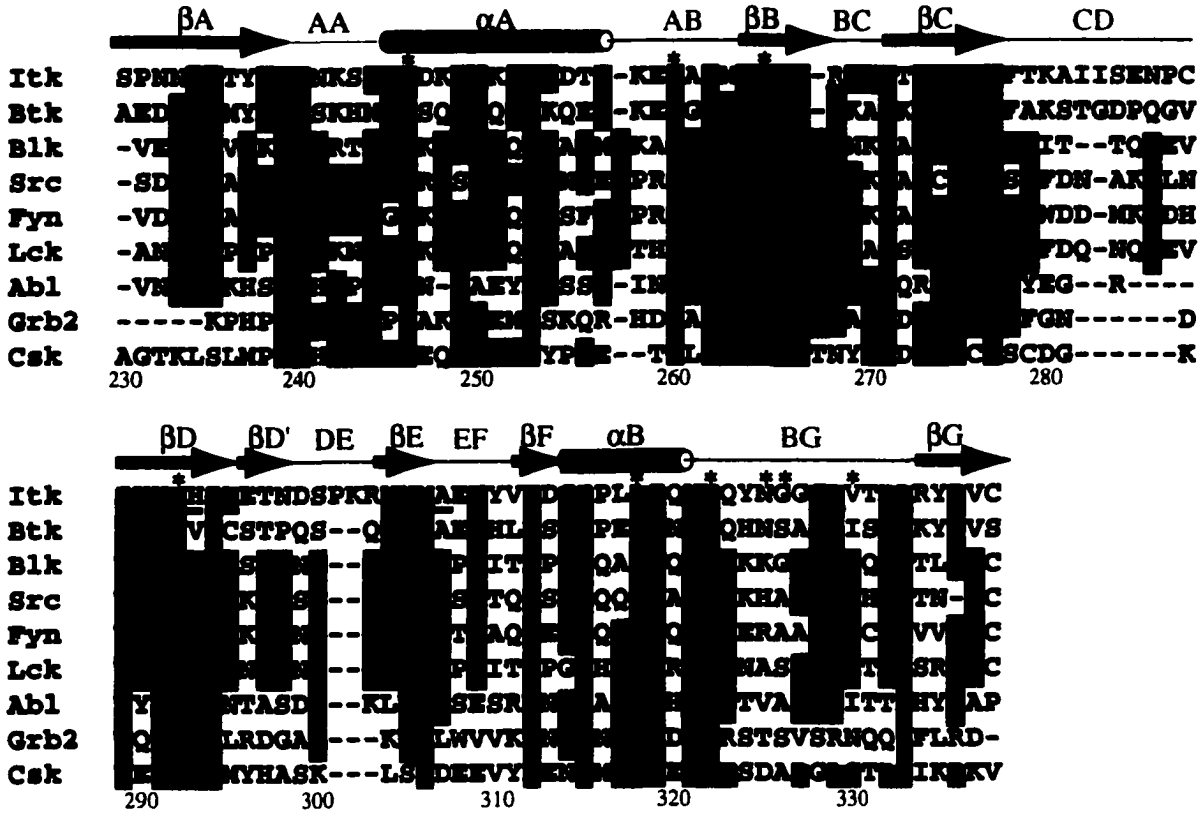

βA AA αA AB βB BC βC CD
Itk
Btk
Blk
Src
Fyn
Lck
Abl
Grb2
Csk
230 240 250 260 270 280
βD βD' DE βE EF βF αB BG βG
290 300 310 320 330

**Figure 5.** Two-dimensional homonuclear NOESY spectra for (A) Itk SH3, (B) equimolar mixture of Itk SH3 and Itk SH2, (C) Itk SH32 dual domain-containing fragment. For each spectrum the region shown includes the indole NH resonance for Trp 208 in the SH3 binding pocket (10.08 ppm for the SH3 domain alone (A) and 10.2 ppm when in the presence of SH2 domain (B&C)). For each of the three samples, intraresidue NOEs to the neighboring aromatics (7.1, 7.3 ppm) and the Trp β protons (2.8, 3.0 ppm) are assigned and indicated by horizontal lines. Additional non-intraresidue NOEs from the Trp208 indole NH are observed in (B) and (C) and are boxed.

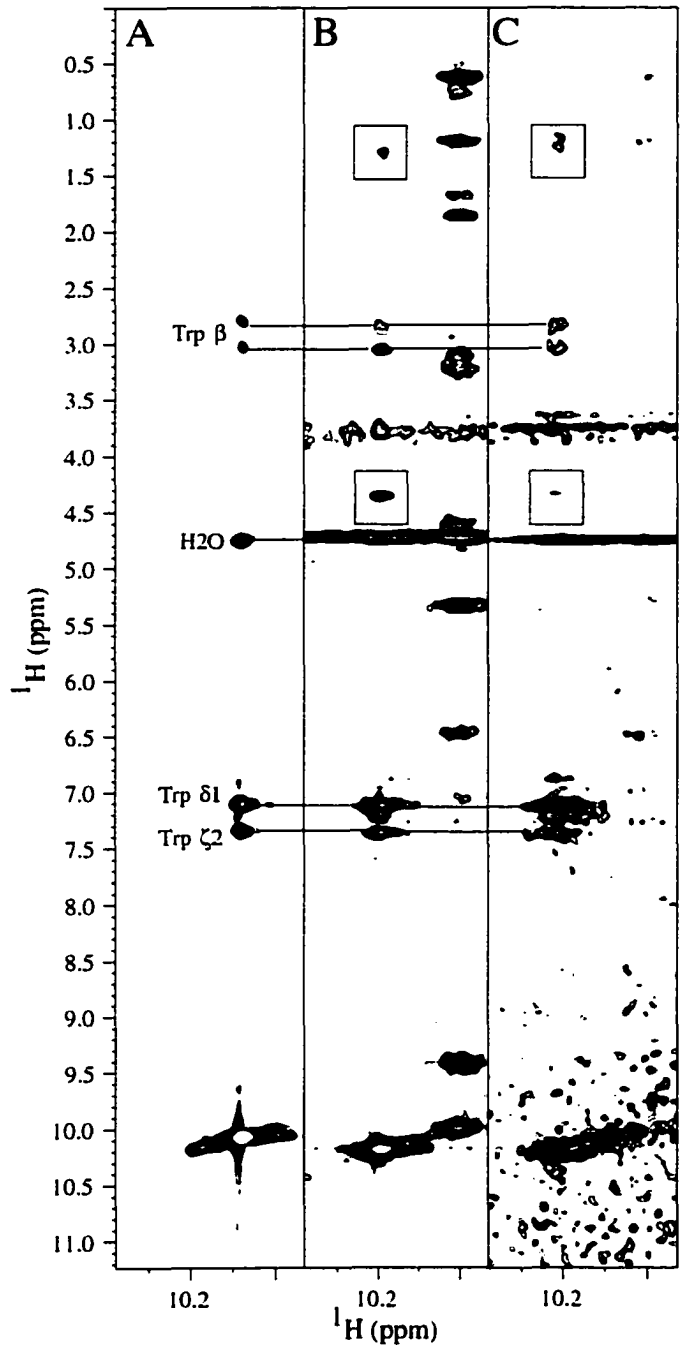

A
B
C
Trp β
H2O
Trp δ1
Trp ζ2
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
10.5
11.0
$^1$H (ppm)
10.2
10.2
10.2
$^1$H (ppm)

**Figure 6.** NMR self-diffusion data for Itk dual domains at 298K. The Itk SH32 and Itk SH3*2(W208K) mutant data are indicated by filled and open circles, respectively. The data are the average values of normalized peak integrals obtained from four replicate experiments. Symbols are as defined in Materials & Methods. The error bars represent three times the standard deviation of the data from the individual experiments. In many cases the error bars are obscured by the data symbols.

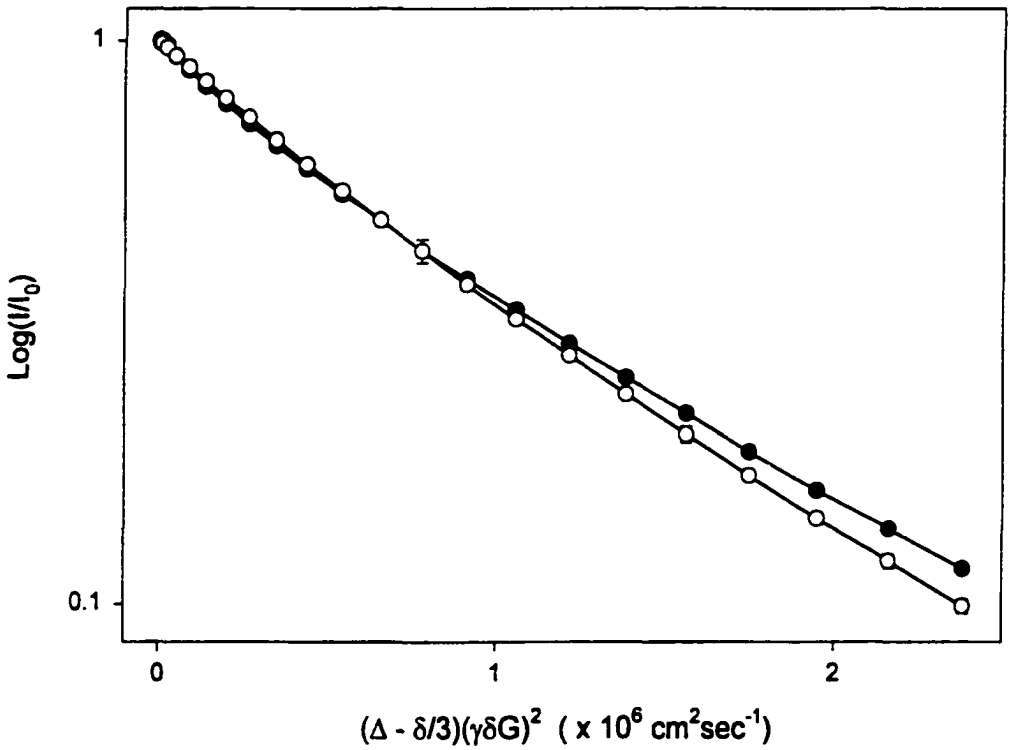

1
0.1
Log(I/I0)
0
1
2
(Δ - δ/3)(γδG)2 ( x 10^6 cm^2sec^-1)

**Figure 7.** Examples of chemical shift titration data used to obtain the binding constant $K_d$ for protein/peptide complexes. Open triangles denote data for residue Y64 in the Itk SH2/ADpYEPPSNDE complex. Closed circles denote data for Y64 in the Itk SH32/ADpYEPPSNDE complex. The solid curves were obtained by fitting the data to Equation 4 via non-linear regression. The estimated $K_d$ for the Itk SH32/ADpYEPPSNDE interaction is $1.78 \pm 1.09$ mM. The large error reflects difficulties in accurately monitoring chemical shift changes due to extensive line broadening associated with the Itk SH32 dual domain fragment.

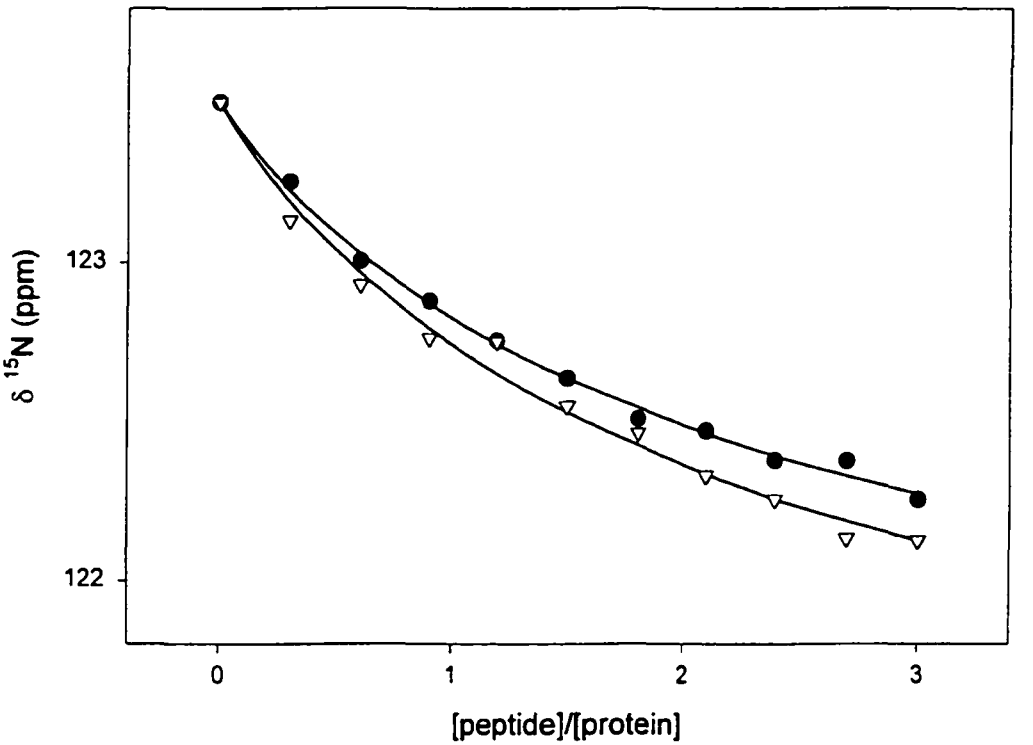
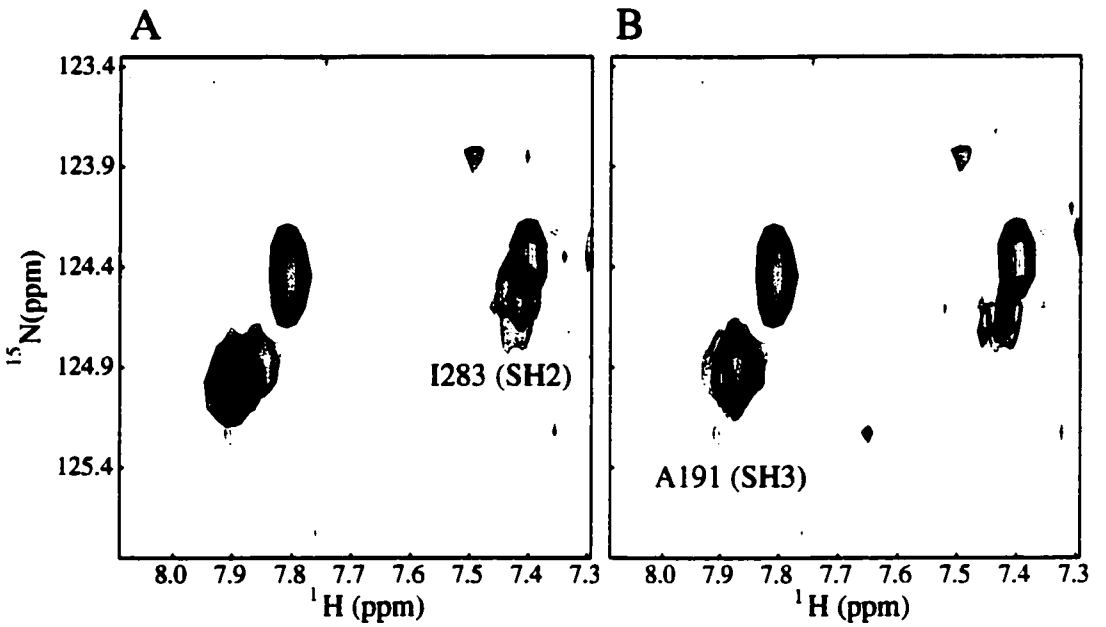

δ 15N (ppm)
123
122
0
1
2
3
[peptide]/[protein]

**Figure 8.** Overlays of three HSQC spectra: *dark blue,* monomeric Itk SH3*2(W208K); *red,* dimeric Itk SH32; *light blue,* Itk SH32 + excess peptide ligand. When comparing (A) and (B), the *dark blue* and *red* spectra are identical, and the difference in the *light blue* spectra is due to addition of either polyproline peptide (SH3 ligand) or phosphotyrosine-containing peptide (SH2 ligand). (A) *light blue* peaks represent Itk SH32 in the presence of 3 fold excess polyproline peptide. To assess the degree to which peptide binding to the SH3 domain causes dimer dissociation, we monitor an SH2 resonance (*e.g.,* I283) that is affected only by changes in the monomer-dimer equilibrium and not peptide association. Peptide binding to the SH3 domain of Itk SH32 dual domain causes SH2 resonances to shift toward the resonance frequency associated with monomer (*dark blue*). (B) Likewise, to assess the affect of phosphotyrosine peptide binding on self-association of Itk SH32, we follow the SH3 domain resonances (*e.g.,* A191). Itk SH32 in the presence of 3 fold excess phosphotyrosine-containing peptide, *light blue,* does not exhibit a significant shift in resonance frequency but rather retains the resonance frequency of the ItkSH32 self-associating dual domain, *red.* It should be noted that the resonance frequencies shown for Itk SH3*2(W208K) are identical to those for the isolated SH3 and SH2 domains and are therefore not affected by the point mutation.


A
B
123.4
123.9
124.4
124.9
125.4
$^{15}$N(ppm)
I283 (SH2)
A191 (SH3)
8.0 7.9 7.8 7.7 7.6 7.5 7.4 7.3
$^{1}$H (ppm)

# CHAPTER 3. Regulation of the tyrosine kinase Itk by the peptidyl-prolyl isomerase cyclophilin A

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Kristine N. Brazin, Robert J. Mallis, D. Bruce Fulton and Amy H. Andreotti

**Abstract**

Interleukin-2 tyrosine kinase (Itk) is a non-receptor protein tyrosine kinase of the Tec family that participates in the intracellular signaling events leading to T cell activation. Tec family members contain the conserved SH3, SH2 and catalytic domains common to many kinase families, but are distinguished by unique sequences outside of this region. The mechanism by which Itk and related Tec kinases are regulated is not well understood. Our studies indicate that Itk catalytic activity is inhibited by the peptidyl prolyl isomerase (PPIase) activity of cyclophilin A (CypA). NMR structural studies combined with mutational analysis show that a proline-dependent conformational switch within the Itk SH2 domain regulates substrate recognition and mediates regulatory interactions with the active site of CypA. CypA and Itk form a stable complex in Jurkat T cells that is disrupted by treatment with cyclosporin A (CsA). Moreover, the phosphorylation levels of Itk and a downstream substrate of Itk, PLC$\gamma$1, are increased in Jurkat T cells that have been treated with CsA. These findings support a novel mode of tyrosine kinase regulation for a Tec

family member and provide a molecular basis for understanding a cellular function of the ubiquitous PPIase, CypA.

**Introduction**

Normal cell growth depends upon the precise control of protein tyrosine kinase activity (1). For certain families of kinases, the mechanism of catalytic regulation is well understood. Structures of Src tyrosine kinases (2,3) reveal intramolecular interactions mediated by the Src homology 2 (SH2) and Src homology 3 (SH3) domains that control catalytic activity of the neighboring kinase domain. Specifically, distortion of the Src kinase active site is achieved in part by SH2 binding to a phosphorylated tyrosine residue in the C-terminal tail of Src (4). For other families of kinases, the mechanistic details of catalytic regulation remain elusive. In particular, the Tec family of non-receptor tyrosine kinases (5) display distinguishing characteristics that point to an alternative mode of regulation. The Tec family kinases modulate hematopoetic cellular responses to external stimuli (6). The T-cell specific Tec family member, Interleukin-2 tyrosine kinase (Itk), (7,8) plays a role in the maturation of thymocytes, is required for intracellular signaling following T cell receptor (TCR) crosslinking, and is involved in generation of second messengers that mediate cytoskeletal reorganization (9). Itk is homologous to Src in the region spanning the SH3, SH2 and kinase domain but lacks the Src C-terminal tail that contains the regulatory tyrosine. However, activation of Itk depends upon SH2-mediated interactions with phosphorylated

signaling partners (9) such as Slp-76 (10) and LAT (11) suggesting a regulatory role for the Itk SH2 domain despite the absence of a Src-like C-terminal regulatory tyrosine residue.

The Src regulatory mechanism highlights the role of molecular switches in controlling cellular signaling pathways. Well-studied covalent modifications such as phosphorylation generate recognition sites that affect the activity and binding behavior of many signaling proteins (1, 12-14). Moreover, there are potential regulatory switches intrinsic to polypeptides such as the *cis/trans* isomerization of peptide bonds preceding the imino acid proline (15,16). In native proteins, the local environment surrounding a proline residue, as well as the overall tertiary structure, influence the relative energies and therefore the relative population of the *cis* and *trans* conformers. The majority of folded proteins for which three-dimensional structural information has been gathered contain *trans* prolyl imide bonds. The *cis* conformation occurs at a frequency of approximately 6% in folded proteins (17) and a small subset of proteins are conformationally heterogeneous with respect to *cis/trans* isomerization (18-21). Furthermore, the activation energy for interconversion between *cis* and *trans* proline is high (~20 kcal/mol) leading to slow interconversion rates (22). This barrier is a rate-limiting step in protein folding and may serve to kinetically isolate two functionally and conformationally distinct molecules. These special properties, and the fact that many proline residues are highly conserved in homologous protein sequences, suggest that prolines may play a role in conformer-specific recognition and function.

Three distinct enzyme families have been identified that catalyze the *cis/trans* isomerization of prolyl imide bonds. These peptidyl-prolyl *cis/trans* isomerases (PPIases)

(23,24) consist of the cyclophilins (CyP), FK506 binding proteins (FKBPs) and the parvulins (25,26). CypA and FKBP12 are the intracellular protein targets of the immunosuppressive natural products CsA and FK506, respectively (27). These small molecules inhibit PPIase activity and block T cell activation. However, the immunosuppressant activity of these small molecules derives not from inhibition of isomerase activity but rather from interruption of signaling events by the inhibitor-PPIase complexes themselves (28). The binary CsA-CypA and FK506-FKBP12 complexes bind to and inhibit calcineurin, the serine/threonine phosphatase responsible for dephosphorylation and subsequent nuclear translocation of the IL-2 transcription factor, nuclear factor of activated T-cells (29,30). The PPIase inhibitors, CsA and FK506, have shed considerable light on immunosuppression as well as the signal transduction pathways involved in T cell signaling, yet the normal cellular roles of the PPIases remain unclear. While recent efforts have identified several PPIase-mediated cellular processes (31-37), the range of normal cellular functions controlled by the ubiquitous PPIases and the molecular basis for their role in regulating signaling cascades have not yet been fully elucidated.

The cellular targets of the PPIases are likely folded proteins that contain a conformationally heterogeneous proline residue. We now present a structural analysis of Itk that places this protein among those that exhibit multiple, well-defined, low energy conformations in solution. A single prolyl imide bond is responsible for the observed conformational heterogeneity within Itk and we show that this proline-dependent conformational switch directly regulates Itk substrate recognition. Moreover, we demonstrate

that the PPIase, CypA, catalyzes the *cis/trans* isomerization of the conformationally heterogeneous prolyl imide bond within Itk. *In vitro* and *in vivo* functional data reveal a stable CypA-Itk complex in T cells and point to a role for CypA in repressing Itk kinase activity. Together, these data support a novel mode of non-receptor tyrosine kinase regulation and provide a molecular basis for understanding a cellular function of the PPIase, CypA.

**Materials and Methods**

**Protein expression and NMR Spectroscopy**. Full-length Itk cDNA (7) for PCR amplification was a gift from Leslie J. Berg (University of Massachusetts Medical School). Protein samples for NMR were expressed and purified as described previously (38). Unless otherwise indicated, all NMR samples were 1.5mM. NMR spectra were recorded at 25°C on a Bruker DRX500 spectrometer operating at a $^{1}H$ frequency of 499.867 MHz. Bovine calf thymus CypA (Sigma) was used for NMR experiments.

***In vitro* kinase assays**. Cell growth conditions, baculovirus infections, and the production of high titer baculovirus stocks were as previously described (39). Sf9 cells (obtained from American Type Culture Collection) were infected with baculovirus expressing Itk (gift from Leslie J. Berg). Harvested cells were lysed in 1% IGEPAL (Sigma) lysis buffer and lysates were cleared by centrifugation at 14,000 rpm for 15 minutes at 4°C. Itk was immunoprecipitated with anti-Itk antibody clone 2F12 (Upstate Biotechnology) and protein

G agarose (GibcoBRL) then washed three times in 50 mM Tris pH 7.6 wash buffer then washed once in kinase buffer (50 mM HEPES buffer pH 7, 10 mM $MgCl_2$, 10 mM $MnCl_2$) before resuspension in ATP/kinase buffer (kinase buffer + 0.5 mM $Na_3VO_4$, 0.5 mM ATP). Kinase reactions were stopped after 30 minutes by addition of 2x SDS loading dye and boiling samples. Samples were resolved on a 10% SDS-polyacrylamide gel, transferred to Immobilon-P membranes (Millipore) and Itk autophosphorylation was detected with anti-phosphotyrosine antibody clone 4G10 (Upstate Biotechnology). Itk was detected with anti-Itk antibody. The level of Itk phosphorylation was analyzed by normalizing detected phosphotyrosine to total Itk in each lane with NIH Image. Recombinant CypA (1 μM, Sigma) and CsA (3 μM, Sigma) were incubated for 6 hours with constant agitation at 4°C. The complex was then centrifuged at 14,000 rpm for 20 minutes to remove any precipitate.

Assay of protein phosphorylation in Jurkat T cells. Jurkat clone E6-1 cells were obtained from ATCC. Cells were maintained at 37°C, 5% $CO_2$ in RPMI medium 1640 (GibcoBRL) supplemented with 10% fetal calf serum (HyClone) and 1x penicillin-streptomycin-glutamine solution (GibcoBRL). 1.25 mg/mL stock solutions of CsA and FK-506 were prepared in DMSO. 2 x $10^7$ Jurkat cells were incubated for 1 hour in the presence of 500 ng/mL of CsA, FK-506 (Calbiochem), or an equal volume of DMSO at 37°C, 5 % $CO_2$. Before stimulation, the cells were washed twice with ice-cold media lacking fetal calf serum and 1x penicillin-streptomycin-glutamine solution containing CsA, FK-506, or DMSO and then resuspended in 400 μL of the same media. Stimulations were performed by incubating the cells on ice for 20 minutes with 10 μg/ mL anti-CD3 antibody (PharMingen), quickly pelleting the cells, and

resuspending in 50 μg/ mL rabbit anti-mouse IgG (Sigma) at 37°C for the indicated times. Stimulations were stopped by diluting the cells 3-fold into ice-cold PBS resuspension buffer (phosphate buffered saline supplemented with 1 mM $Na_3VO_4$ and 20 mM NaF). Cells were pelleted and resuspended in ice-cold RIPA lysis buffer, lysates were cleared by centrifugation. Cleared lysates were depleted of remaining antibodies used during the stimulation by incubation with protein G agarose for 1 hour at 4°C with constant rocking. Either Itk, Zap-70 or PLCγ1 were immunoprecipitated from pre-cleared lysates with either anti-Itk antibody, anti-Zap-70 antibody (Upstate Biotechnology), or anti-PLCγ1 antibody clone B-6-4 (Upstate Biotechnology) and protein G or protein A agarose. Immunoprecipitates were washed 3 times in RIPA lysis buffer and once in PBS resuspension buffer. Samples were resolved on an 8% or a 12.5% (for CypA detection) SDS-polyacrylamide gel. Itk detection was as described above and Zap-70 and PLCγ1 were detected with anti-Zap-70 antibody and anti-PLCγ1 antibody, respectively. CypA was detected with anti-CypA (Calbiochem), and FKBP12 was detected with anti-FKBP (N-19) (Santa Cruz Biotechnology, Inc.). The identity of the CypA band was confirmed by co-migration with purified protein purchased from Sigma.

**Competitive binding assays.** Lysates and immunoprecipitated Itk were obtained as described above from unstimulated Jurkat T cells. The protein G agarose, containing immunoprecipitated Itk, was briefly centrifuged and the supernatant was removed. The agarose was then resuspended in 100 μL of RIPA cell lysis buffer that contained 50 μg of the

indicated ligand. After 1 hour of incubation at 4°C with constant rocking, the agarose was washed 3 times in RIPA lysis buffer and once in PBS resuspension buffer before resuspension in 20 μL of 2x SDS loading dye. Samples were resolved on a 12.5% SDS-polyacrylamide gel. Detection of CyP A and Itk were as described above. Itk GST-SH3 was detected with anti-GST (Upstate Biotechnology). The level of CypA co-immunoprecipitation was analyzed by normalizing detected CypA to total Itk in each lane using NIH Image. Synthesis of the ADpYEPP and QQPPVPPQRPMA peptides was carried out by Gautam Sarath at the University of Nebraska Lincoln Protein Core Facility.

## Results

**Proline isomerization induces conformational heterogeneity within the Itk SH2 domain.** Structural analysis of the purified Itk SH2 domain shows two well-defined, low energy conformations are populated in solution (Fig. 1a). NMR resonance assignments for the $^{15}$N-labeled Itk SH2 domain reveal two sets of resonances in slow exchange for 35 of the 109 SH2 residues. Characteristic NOE correlations (40) between Asn-286 and Pro-287 indicate that proline *cis/trans* isomerization around the Asn-286-Pro-287 imide bond is the source of the conformational heterogeneity. A heteronuclear single quantum correlation (HSQC) (41) spectrum of the Itk SH2 domain in which Pro-287 is mutated to Gly (Fig. 1b) confirmed this assessment. For the P287G mutant, in which the 286-287 amide bond adopts only the *trans* conformation, all of the $^{1}$H-$^{15}$N crosspeaks assigned to the *cis* conformer are absent. For many of the SH2 residues in the wild type protein, the differences in chemical

shift values between the *cis* and *trans* conformers are large (>6ppm in the $^{15}N$ dimension and >1ppm in the $^{1}H$ dimension), reflecting significant differences in the chemical environment of nuclei in the two forms (Fig. 1a). The largest chemical shift differences between the *cis* and *trans* conformers occur for residues surrounding Pro-287 in primary amino acid sequence. However, it is notable that large chemical shift differences are also apparent in regions removed in primary sequence from Pro-287, in particular, residues located within the C-terminus of the SH2 domain (residues 329-335). Together, the conformationally heterogeneous regions map to a contiguous surface of the Itk SH2 domain (Fig. 2a,b).

**The *cis* and *trans* proline conformers bind different ligands.** We have previously demonstrated that the Itk SH2 domain mediates two distinct binding events: canonical recognition of phosphotyrosine-containing peptides and phosphotyrosine-independent dimerization via contacts to the Src homology 3 (SH3) binding pocket of another Itk molecule (38). Chemical shift mapping reveals the contiguous SH2 surface residues that are involved in binding to either the Itk SH3 domain (Fig. 2a) or phosphopeptide (Fig. 2b). The overlap between the SH3 and phosphopeptide binding surfaces is within the conformationally heterogeneous region of the Itk SH2 domain. This suggests that the conformational heterogeneity induced by proline isomerization plays a role in regulating ligand selection. Indeed, addition of excess Itk SH3 domain to the SH2 domain shifts the *cis/trans* equilibrium to favor the *cis* SH2 conformer (Fig. 2c). As well, shifts in the positions of crosspeaks corresponding to the *cis* proline conformer but not those of the *trans* SH2 conformer are

observed in the HSQC spectrum of the Itk SH2 domain to which recombinant Itk SH3 domain has been added (Fig. 2d). Thus, the *cis* Asn-286-Pro-287 imide bond, and not the *trans*, is required for Itk dimerization. In contrast, a phosphotyrosine-containing peptide binds preferentially to the *trans* SH2 conformer. Addition of excess phosphopeptide to the Itk SH2 domain shifts the equilibrium to favor the *trans* conformer (Fig. 2e). Chemical shift changes are observed for crosspeaks corresponding to some residues in the *trans* SH2 conformer but not for those same residues in the *cis* (Fig. 2f). This demonstrates conformer-specific ligand recognition mediated entirely by isomerization of a single imide bond.

**The Itk SH2 domain is a substrate for the PPIase, CypA.** NMR linewidths (42) were examined in spectra of $^{15}$N-labeled SH2 domain in the presence of substoichiometric amounts of two different PPIases (CypA and FKBP-12). Line-broadening of the conformationally heterogeneous resonances within the Itk SH2 domain was observed upon addition of 3 mol% and 20 mol% CypA (Fig. 3). In contrast, the spectrum of the Itk SH2 domain incubated with 13 mol% FKBP-12 was identical to that of SH2 domain alone (data not shown). Addition of CypA to the Itk SH2 domain also caused small increases in all linewidths and specific chemical shift perturbations that were localized to residues surrounding Pro-287. The P287G mutation within the Itk SH2 domain abrogates interaction with CypA (data not shown). Together, these data provide evidence for a direct, specific, and productive interaction between CypA and a *folded* domain of a protein tyrosine kinase. By lowering the kinetic barrier to interconversion, CypA accelerates *cis/trans* isomerization and

may play an integral role in a coupled equilibrium system involving conformer-specific recognition (Fig. 3d). Such a mechanism would allow Itk to switch binding partners more rapidly in response to exogenous signaling events.

**CypA is an inhibitor of Itk kinase activity.** Given the fact that CypA catalyzes the *cis/trans* isomerization within the Itk SH2 domain, we carried out *in vitro* functional assays to examine the role of CypA in regulating Itk activity. Itk was expressed in Sf9 cells with the baculovirus expression system and purified by immunoprecipitation. Following incubation with ATP, *in vitro* Itk autophosphorylation (39) was detected by Western blotting. Itk autophosphorylation was decreased significantly in the presence of CypA (Fig. 4a). CsA reversed the effect of CypA on Itk kinase activity (Fig. 4a). Thus, CypA inhibits Itk kinase activity *in vitro*.

To assess the role of cyclophilin in regulating Itk activity *in vivo*, the level of Itk phosphorylation was monitored following TCR/CD3-induced stimulation of Jurkat T cells that were either untreated or treated with CsA. Itk tyrosine phosphorylation, as monitored by Western blotting, was apparent at early timepoints following TCR stimulation. The level of Itk phosphorylation in untreated cells increased after a 40 second stimulation and then decreased toward basal levels after a 2 minute stimulation (Fig. 4b). In the CsA treated cells, the level of Itk phosphorylation was higher than the untreated cells at both timepoints (Fig. 4b). Additionally, endogenous CypA was detected in Itk immunoprecipitates prepared from Jurkat T cells that have not been treated with CsA indicating the presence of a stable Itk-

CypA complex in T cells (Fig. 4c). In Itk immunoprecipitates of cells pre-treated with CsA, the amount of CypA was less than in untreated cells (Fig. 4c). The effect of CypA on Itk phosphorylation is specific since FK-506 treatment of Jurkat cells had no detectable effect on Itk phosphorylation (Fig. 4d) and FKBP-12 was not detected in Itk immunoprecipitates of untreated cells or cells treated with FK-506 (data not shown). This observed specificity is consistent with NMR experiments that reveal that CypA, but not FKBP, affects the *cis/trans* isomerization within the Itk SH2 domain (Fig. 3). Since both CsA and FK-506 treatment lead to inhibition of calcineurin and subsequent immunosuppression (29,30), the absence of an FK-506 mediated effect on Itk phosphorylation levels indicates that the increase in Itk phosphorylation upon treatment with CsA (Fig. 4b) is the direct result of inhibition of CypA activity and is not related to the immunosuppressive action of CsA.

**CsA treatment affects the phosphorylation state of PLCγ1 but not Zap-70 in Jurkat T cells.** Phosphorylation of the upstream and downstream signaling partners of Itk, Zap-70 and PLCγ1, respectively (43-45) was monitored following TCR/CD3-induced stimulation of Jurkat T cells carried out in the presence and absence of CsA. Zap-70 phosphorylation levels at both 40 second and 2 minute stimulations are unchanged by treatment with CsA (Figure 4e) consistent with the placement of Zap-70 upstream of Itk. In contrast, an *increase* in the level of PLCγ1 phosphorylation in a manner that parallels Itk is observed following CsA treatment of Jurkat cells. PLCγ1 phosphorylation is higher after both the 40 second and 2 minute stimulations in CsA treated cells as compared to the same

timepoints from untreated cells (Fig. 4f). Therefore, Zap-70, a signaling molecule upstream of Itk is not affected by CsA treatment whereas, PLCγ1, a signaling molecule downstream of Itk is up-regulated by CsA treatment. These data suggest that an increase in Itk activity resulting from inhibition of CypA leads to a concomitant increase in downstream PLCγ1 activity.

**The Itk SH2 domain mediates CypA binding in T cells.** Competitive binding assays with ligands that specifically target the Itk SH2 domain provide evidence that the Itk-CypA interaction in Jurkat T cells is mediated by the Itk SH2 domain. The presence of two different SH2 ligands in Itk immunoprecipitates from Jurkat T cells Itk SH3 domain (38) or the phosphopeptide (ADpYEPP, denoted pTyr)) decreased the amount of co-immunoprecipitated CypA (Fig. 5, *i*). In contrast, the presence of a peptide ligand that binds specifically to the Itk SH3 domain (QQPPVPPQRPMA, denoted PXXP) caused no detectable change in the amount of co-immunoprecipitated CypA (Fig. 5, *i*). Consistent with our previous structural analysis of Itk dimerization (38), a significant amount of Itk GST-SH3 was detected following washing of Itk immunoprecipitates that have been incubated Itk GST-SH3 (Fig. 5, *iii*). This observation is consistent with the presence of the *cis* SH2 conformer in full-length Itk from Jurkat T cells. Taken together, *in vitro, in vivo* and NMR data implicate CypA as a regulator of Itk activity in T cells and indicate that P287 within the Itk SH2 domain mediates critical regulatory interactions with CypA.

**Discussion**

Our data suggest that displacement of CypA activates Itk. Phospholigand binding and Itk dimerization both compete with CypA binding to the Itk SH2 domain (Fig. 5). A mechanistic model for Itk activation (Fig. 6) can be proposed based on the well understood structural details of SH2-phospholigand recognition (46). The SH2 domain consists of two adjacent binding pockets: the 'pY' pocket that contacts the phosphotyrosine residue itself and the 'pY+3' pocket that confers ligand-binding specificity by contacting amino acids flanking the phosphotyrosine. Unlike the Itk pY+3 pocket, the pY binding site is not affected by proline-induced conformational heterogeneity (Fig. 2a,b), nor does it appear, based on the lack of chemical shift perturbations, to be sterically occluded by CypA binding. Itk activation by a phospholigand may therefore be initiated by binding of the phosphotyrosine itself to the accessible pY pocket in the SH2 domain. Subsequent pY+3 engagement would displace CypA and stabilize the active, *trans* SH2 conformer (Fig. 6). In fact, phospholigand binding to the SH2 domain has previously been shown to activate Itk (45,47). Our data indicate that Itk dimerization also causes displacement of CypA (Fig. 5) but results in stabilization of the *cis* SH2 conformer for which functional data have not yet been obtained. Nevertheless, the activity of Itk appears to correlate with the identity of SH2 bound ligand and therefore the configuration of the Asn-286-Pro-287 imide bond.

At this time, the relative affinities of the Itk-CypA interaction, SH3/SH2 mediated Itk dimerization, and Itk SH2-phospholigand binding in a cellular context are not known. Certainly, the presence of CypA in immunoprecipitates of Itk suggests that it is a stable

binding partner of Itk in T cells. NMR data show that CypA binds to the Itk SH2 domain and catalyzes the *cis/trans* isomerization of the N286-P287 imide bond. These two observations suggest two possible modes of action *in vivo*. CypA (as a binding partner) may act as a repressor of Itk activity by stabilizing a transition-state conformation (48) around the N286-P287 imide bond within Itk (Fig. 6). Alternatively, CypA (as a catalyst) may facilitate rapid interconversion between the *trans* and *cis* Itk conformers and therefore between the phospholigand bound and dimerized states, respectively (Fig. 3d). In any case, structural rearrangements in the SH2 domain due to dimerization, CypA or phospholigand binding could allosterically regulate the neighboring kinase domain, in a manner analogous to the established conformational regulation of the Src kinases (2,3). The specific interactions mediated by the regulatory domains in the Tec family kinases are different from the Src family kinases, yet the ability of these domains to control the catalytic activity through conformational changes in the kinase domain may be similar.

To date, detailed mechanistic studies of PPIase function have been limited to analysis of peptidyl-prolyl isomerization within small peptide model systems. We have identified a folded protein target for CypA that provides the opportunity to study the PPIase catalytic mechanism in the context of a physiological protein substrate. An analysis of the extent to which the tertiary structure of a physiological substrate affects CypA activity is now tractable and comparison with structures such as the CypA-HIV capsid protein complex (49) should shed additional light on the mode of action of CypA. Moreover, the past decade has witnessed an enormous effort to elucidate the mode of action of the immunosuppressive

agents, CsA and FK-506. This work has advanced our understanding of T cell signaling and our ability to control the immune response. While these natural products target the active sites of cyclophilin and FKBP, the fact that they inhibit PPIase activity is apparently irrelevant to their immunosuppressive effects. The discovery of a non-receptor protein tyrosine kinase as a physiological target of CypA in T cells raises the possibility that other conformationally heterogeneous kinases exist that are regulated by PPIases in a similar manner. These findings are provocative in light of the high incidence of cancer that accompanies the use of CsA in organ transplant patients (50). Hyperactivation of protein tyrosine kinases, including Itk, during clinical use of PPIase inhibitors may contribute to unregulated cellular proliferation and ultimately formation of malignancies in immunosuppressed patients. Certainly, further exploration of the physiological roles of the PPIases is warranted and may provide a framework upon which more selective immunosuppressive therapies may be developed.

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**Fig. 1.** The Itk SH2 domain adopts two stable structures in solution. (a) $^{1}H$-$^{15}N$ HSQC spectrum of purified, recombinant $^{15}N$-labeled Itk SH2 domain. The resonances corresponding to the *cis* (*c*) and *trans* (*t*) conformers are connected by a gray double-headed arrow. The inset is an expansion of the boxed region of the spectrum containing representative doubled resonances for the Asn-325 side chain (sc) $NH_2$ and the Lys-258 backbone NH. (b) HSQC spectrum of the Itk SH2 domain in which Pro-287 is mutated to Gly. Labeled crosspeaks correspond to those that are labeled in (a) and the crosspeak corresponding to Gly-287 (introduced by mutation) is assigned. Inset is the same region as in (a) but shows a single resonance for Asn-325(sc) and Lys-258.

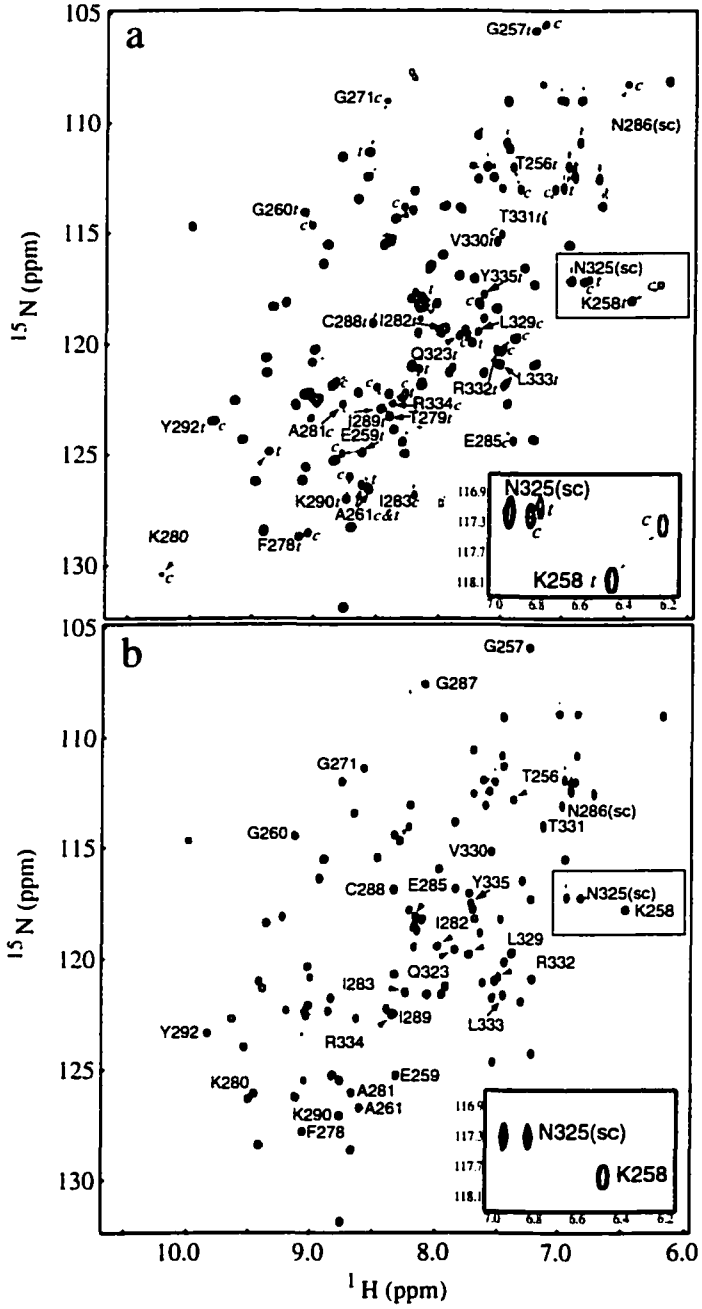

a
b
$^{15}$N (ppm)
$^{1}$H (ppm)
105
110
115
120
125
130
10.0
9.0
8.0
7.0
6.0
G257
G287
G271
T256
N286(sc)
T331
G260
V330
C288
E285
Y335
N325(sc)
K258
I282
L329
R332
I283
Q323
I289
L333
Y292
R334
E259
K280
A281
A261
K290
F278
N325(sc)
K258

**Fig. 2.** Surface representations (rendered with MolMol (51)) of the Itk SH2 *cis* (a) and *trans* (b) structural models (R.J.M. and A.H.A., unpublished results). The SH2 residues that display conformational heterogeneity are demarcated with a dotted line on each model. Pro-287 is indicated and the pY and pY+3 binding pockets are labeled. Chemical shift perturbations that occur upon addition of equimolar Itk SH3 domain to the Itk SH2 domain (38) are mapped onto the *cis* model and highlighted in gray (a). Likewise, SH2 residues that undergo chemical shift perturbations upon addition of three times excess phosphopeptide (ADpYEPPPSNDE) are highlighted in yellow on the *trans* model (b). For both surface models, residues that do not exhibit chemical shift changes upon addition of ligand are orange. (c) Select region of the HSQC spectrum of a 0.5mM Itk SH2 sample *left*, Itk SH2 domain alone where the *trans/cis* ratio is approximately 60:40 based on the crosspeak volumes for each set of doubled resonances. *right*, The same region of the Itk SH2 HSQC spectrum after addition of 10 times excess unlabeled Itk SH3 domain (*trans/cis* ratio 10:90). (d) Superposition of the same region of two HSQC spectra of $^{15}$N-labeled Itk SH2 domain in the absence (black) and presence (gray) of unlabeled Itk SH3 domain. The Thr-331 *cis* crosspeak shifts upon addition of equimolar Itk SH3 domain (arrow) while the Thr-331 *trans* crosspeak remains at the same frequency. (e) *left*, Itk SH2 domain (*trans/cis* 60:40) *right*, Addition of 3 times excess phosphopeptide to Itk SH2 (*trans/cis* 90:10). (f) Superposition of two HSQC spectra of $^{15}$N-labeled Itk SH2 domain in the absence (black) and presence (gray) of 3 times excess phosphopeptide. The Ala-261 and Ala-281 *trans* crosspeaks shift upon addition of phosphopeptide (arrows) while the Ala-261 and Ala-281 *cis* crosspeaks resonate

at the same frequency regardless of ligand present. (The Ala-281*cis* crosspeak is shown in the inset due to the large difference in resonance frequencies for the *cis* and *trans* resonances of Ala-281.)

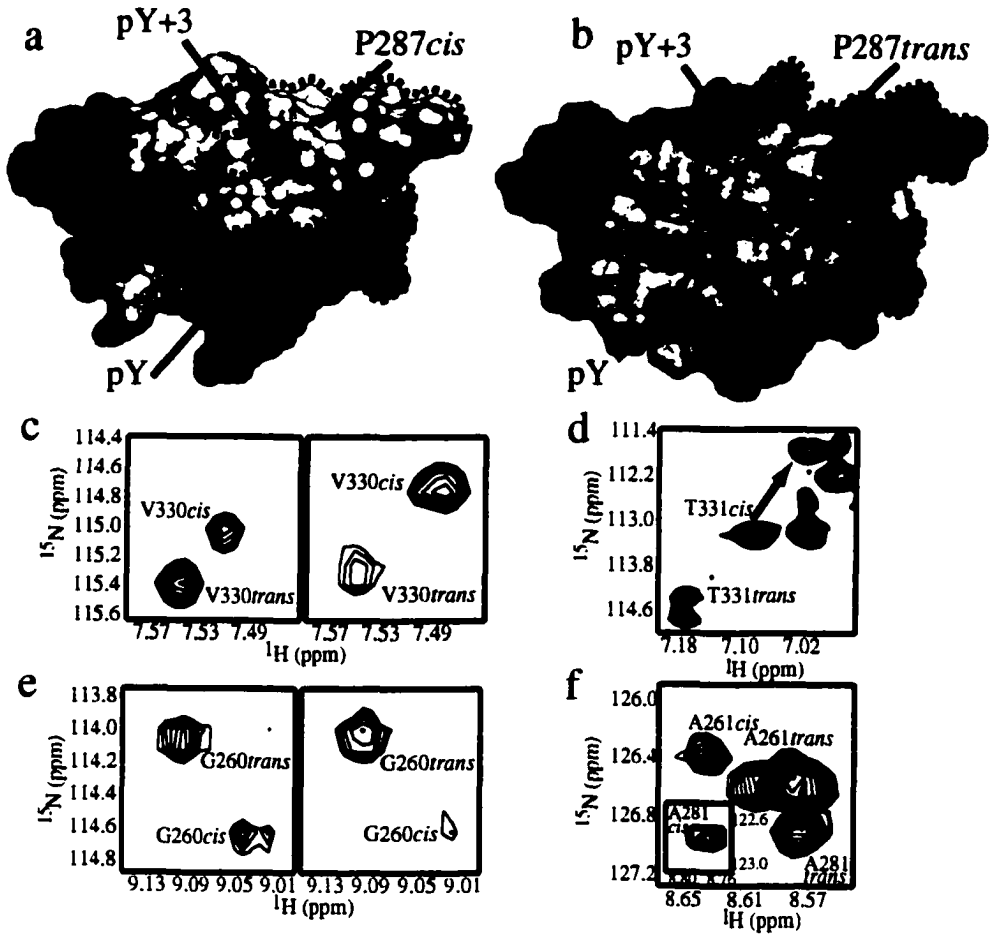
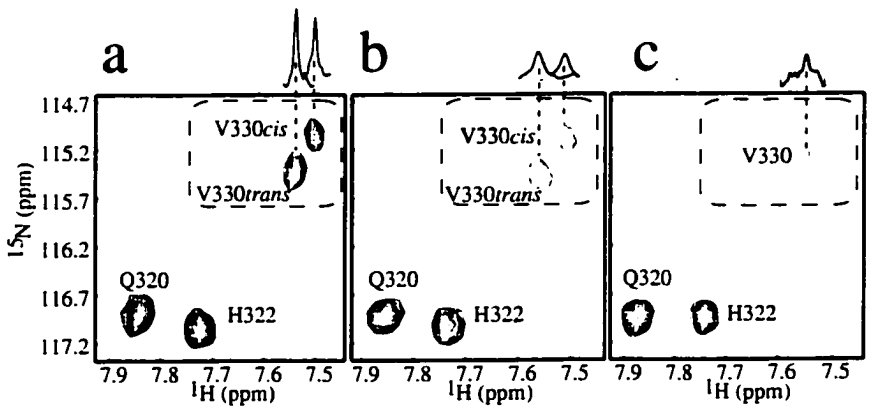

a
pY+3
P287cis
pY
b
pY+3
P287trans
pY
c
V330cis
V330trans
V330cis
V330trans
15N (ppm)
1H (ppm)
d
T331cis
T331trans
15N (ppm)
1H (ppm)
e
G260trans
G260cis
G260trans
G260cis
15N (ppm)
1H (ppm)
f
A261cis
A261trans
A281
22.6
23.0
A281
15N (ppm)
1H (ppm)

**Fig. 3.** (a) Region of the HSQC spectrum for the $^{15}$N-labeled Itk SH2 domain (0.5 mM) that includes crosspeaks for the Gln-320, His-322 and Val-330 backbone NH resonances. Gln-320 and His-322 are not affected by *cis/trans* isomerization around the Asn-286-Pro-287 imide bond and therefore each appears as a single crosspeak. In contrast, the Val-330 amide resonance is doubled as a result of slow exchange between the *cis* and *trans* forms. (b) Same region of the HSQC spectrum for the Itk SH2 domain (0.5 mM) in the presence of 3 mol% CypA. Line broadening is apparent for all of the doubled resonances in the SH2 domain spectrum upon addition of CypA, while those peaks that correspond to residues unaffected by the isomerization event do not broaden significantly. (c) Addition of 20 mol% CypA to the Itk SH2 domain results in further line broadening and coalescence to the chemical shift value that represents the average of the *cis* and *trans* conformers. One-dimensional projections through the center of the Val-330 crosspeaks along the $^{1}$H axis illustrate the exchange mediated broadening and coalescence. (d) Equilibrium model encompassing the CypA catalyzed Itk SH2 *cis/trans* interconversion, *cis* mediated SH3 binding (Itk dimerization) and *trans* mediated phospholigand (pY) binding.


a
b
c
114.7
115.2
115.7
116.2
116.7
117.2
$^{15}$N (ppm)
V330cis
V330trans
V330
Q320
H322
7.9 7.8 7.7 7.6 7.5
$^{1}$H (ppm)

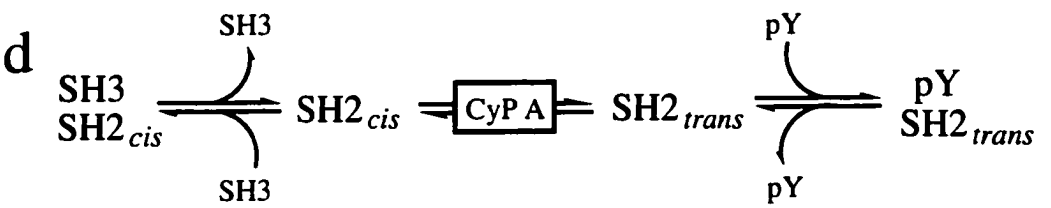

d
SH3
SH3
SH2 cis
SH2 cis
CyP A
SH2 trans
pY
pY
SH2 trans

**Fig. 4.** (a) CypA, CypA/CsA complex, or CsA were added to baculovirus expressed Itk immediately prior to resuspension in ATP/kinase buffer. A mock kinase assay of Itk (left lane) was performed in which ATP was excluded from the kinase reaction buffer. Itk phosphorylation is normalized to Itk protein levels and net changes in phosphorylation are indicated below each lane. For (b) through (f) lanes 1, 2 and 3 are mock stimulation, 40 seconds and 2 minutes, respectively, following TCR stimulation of Jurkat cells in the absence (-) of drug treatment while lanes 4, 5 and 6 represent the same conditions in the presence (+) of drug. (b) Itk phosphorylation following TCR stimulation of Jurkat cells in the presence and absence of CsA. (c) CypA binding to Itk was monitored by detection of Itk immunoprecipitates with an anti-CypA antibody. (d) Itk phosphorylation in the presence and absence of FK-506. (e) Zap-70 phosphorylation and (f) PLC$\gamma$1 phosphorylation in the presence and absence of CsA. Phosphorylation levels of Itk, Zap-70 and PLC$\gamma$1 are normalized to total protein in each lane. Net changes in phosphorylation relative to phosphorylation levels at 40 seconds following stimulation in the absence of drug (lane 2) are indicated below each lane.

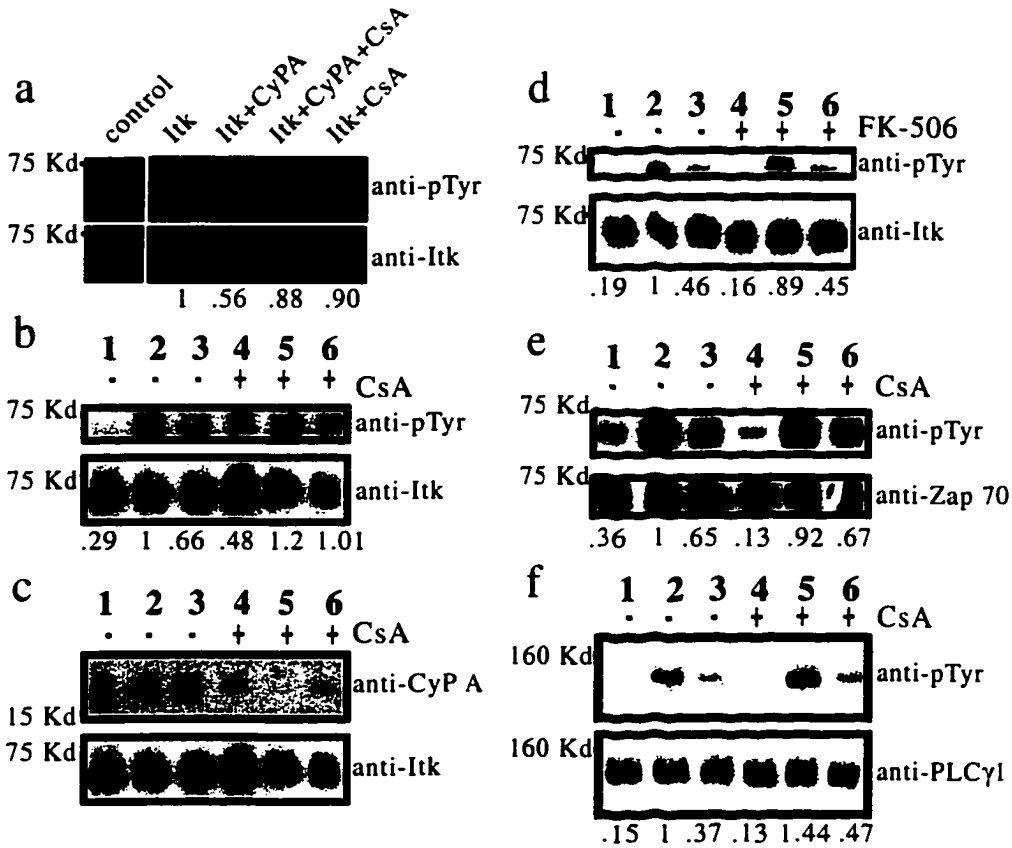

a
control
Itk
Itk+CyPA
Itk+CyPA+CsA
Itk+CsA
75 Kd
anti-pTyr
75 Kd
anti-Itk
1 .56 .88 .90
b
1 2 3 4 5 6
CsA
75 Kd
anti-pTyr
75 Kd
anti-Itk
.29 1 .66 .48 1.2 1.01
c
1 2 3 4 5 6
CsA
anti-CyP A
15 Kd
75 Kd
anti-Itk
d
1 2 3 4 5 6
FK-506
75 Kd
anti-pTyr
75 Kd
anti-Itk
.19 1 .46 .16 .89 .45
e
1 2 3 4 5 6
CsA
75 Kd
anti-pTyr
75 Kd
anti-Zap 70
.36 1 .65 .13 .92 .67
f
1 2 3 4 5 6
CsA
160 Kd
anti-pTyr
160 Kd
anti-PLCγ1
.15 1 .37 .13 1.44 .47

**Fig. 5.** For the indicated samples, GST, Itk GST-SH3, phosphotyrosine-containing peptide (pTyr), or a proline-rich peptide (PXXP) were incubated with full-length Itk following immunoprecipitation from Jurkat T-cell lysates. Following extensive washing, the amount of CypA that co-immunoprecipitated with Itk was determined by immunoblotting (*Top, i*). The amount of CypA in Itk immunoprecipitates is normalized to total Itk in each lane. Net changes in the amount of co-immunoprecipitated CypA relative to the amount that co-immunoprecipitates with Itk in the absence of exogenous factors are indicated. (*Bottom, iii*) Detection of GST-SH3 in Itk immunoprecipitates following competitive binding assay.

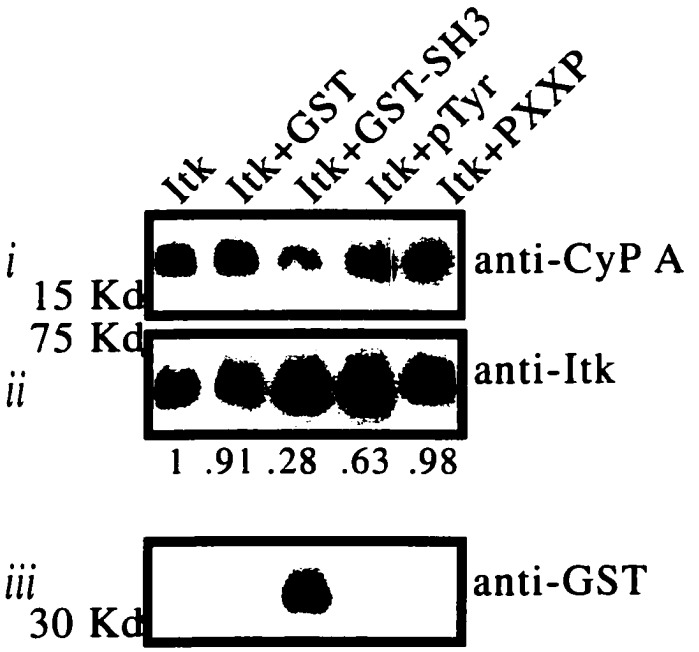
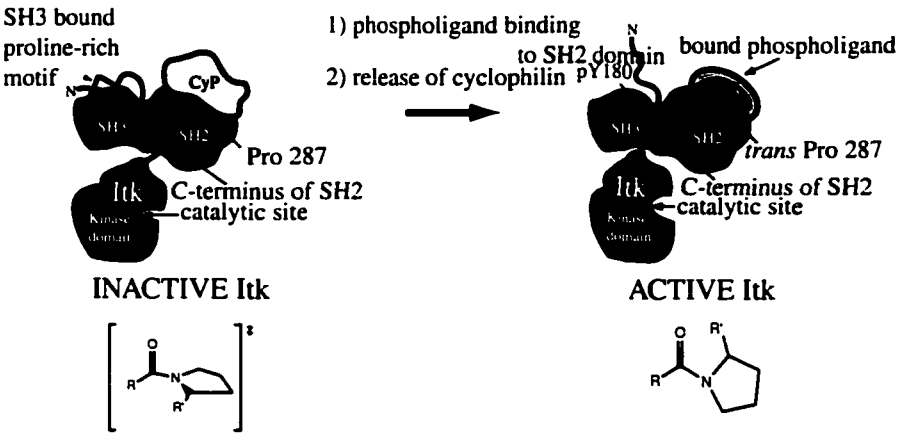

Itk
Itk+GST
Itk+GST-SH3
Itk+pTyr
Itk+PXXP
i
15 Kd
anti-CyP A
75 Kd
ii
anti-Itk
1 .91 .28 .63 .98
iii
30 Kd
anti-GST

**Fig. 6.** Model for Itk regulation. Itk is dark gray and includes the proline-rich region, SH3, SH2 and kinase domains (the PH domain and part of the TH domain are not shown for clarity). Two 'states' of Itk are depicted: inactive, CypA-bound Itk and active, phospholigand-bound Itk. CypA is light gray and is shown bound to the Itk SH2 domain in the region of Pro-287. The proposed configuration of the Asn-286-Pro-287 imide bond (resembling the transition state for *cis/trans* interconversion) is illustrated for the inactive, CypA bound form of Itk. NMR spectroscopic data suggest that cyclophilin-bound Itk is monomeric (unpublished results, K.N.B. & A.H.A.) and the proline-rich region may contact the SH3 binding pocket in an intramolecular fashion (52). Activation may be accompanied by phosphorylation of Y180 in the SH3 domain expelling bound proline (53). *Cis/trans* isomerization around Asn-286-Pro-287 causes pronounced conformational heterogeneity in the C-terminus of the Itk SH2 domain leading directly into the Itk kinase domain (see Fig. 1). The C-terminus of the Itk SH2 domain may adopt a conformation in the presence of cyclophilin that structurally perturbs the kinase catalytic site rendering it inactive. Release of cyclophilin, either by Itk dimerization to favor the *cis* conformer (not shown) or by binding of a phosphotyrosine-containing ligand to favor the *trans* conformer may lead to reorganization of the C-terminal SH2 residues and subsequent restructuring of the kinase active site.


SH3 bound
proline-rich
motif
CyP
SH2
Pro 287
Itk
C-terminus of SH2
catalytic site
INACTIVE Itk
1) phospholigand binding
to SH2 domain
2) release of cyclophilin
bound phospholigand
trans Pro 287
Itk
C-terminus of SH2
catalytic site
ACTIVE Itk

# CHAPTER 4. Proline *cis/trans* isomerization governs the quaternary structure of the T cell specific tyrosine kinase Itk

A paper submitted to the *Journal of Molecular Biology* (2002).

Kristine N. Brazin, D. Bruce Fulton and Amy H. Andreotti

## Summary

We have examined the role of proline *cis/trans* isomerization in determining the quaternary structure of the T-cell specific tyrosine kinase, Interleukin-2 tyrosine kinase (Itk). Two distinct structural arrangements have been previously described for the non-catalytic domains of Itk. An intermolecular self-association mediated by a specific interaction between the Itk Src homology 2 (SH2) and Src homology 3 (SH3) domains results in dimerization of SH2- and SH3-containing fragments. As well, the structure of an intramolecular complex between the SH3 domain and the adjacent proline-rich stretch within Itk has been solved previously. Both intermolecular SH2 binding and intramolecular binding of the Itk proline-rich region require the conserved binding pocket of the Itk SH3 domain and are therefore mutually exclusive. We now demonstrate that *cis/trans* isomerization around the imide bond preceding the proline residue at position 287 (P287) in the Itk SH2 domain determines which ligand binds to the adjacent SH3 domain. The *cis* conformation of the prolyl 287 imide bond is required for intermolecular contacts to an opposing SH3 domain and therefore Itk dimer formation. Mutation of the conformationally heterogeneous P287 to a glycine results in formation of the previously described intramolecular interaction between the SH3 domain and

the proline-rich region. Thus, proline isomerization serves as a molecular switch that regulates the quaternary structure of Itk.

**Introduction**

Inspection of the domain structure of protein kinases reveals the presence of non-catalytic domains outside of the catalytic core. In particular, SH2 and SH3 domains are adjacent to the catalytic domain in numerous kinases.[1,2] For the Src family of tyrosine kinases, x-ray crystallographic analyses have clearly defined the role of these non-catalytic domains in controlling kinase activity.[3,4,5] An intramolecular interaction between the SH3 domain and the linker sequence preceding the kinase domain as well as an intramolecular contact between the Src SH2 domain and a phosphorylated tyrosine in the carboxy-terminal tail are critical for inhibition of Src catalytic activity. Mutation or deletion of the non-catalytic regulatory domains of Src leads to constitutive activity that in a cellular context promotes aberrant cellular signaling and results in uncontrolled cell growth.[6,7,8] The second largest class of SH3 and SH2-containing tyrosine kinases, the Tec family kinases, modulate hematopeotic cellular responses to external stimuli.[9,10] The Tec kinases share sequence homology with the Src kinases in the region that spans the SH3, SH2 and kinase domains but contain two distinct, defining domains (Pleckstrin homology (PH) and Tec homology (TH)) and lack the carboxy-terminal regulatory sequence critical to Src regulation. The regulatory mechanisms of the Tec family kinases are not known. However, differences in the domain

structures between the Src and Tec kinases demand distinct mechanisms of regulation for the Tec family.

The T cell specific Tec family member, Interleukin-2 tyrosine kinase (Itk),[11,12,13] is an important component of antigen receptor signaling pathways.[14,15,16] To date, structural characterization of Itk has been limited to two different Itk fragments. First, structure determination using nuclear magnetic resonance (NMR) spectroscopy revealed an intramolecular interaction between the binding cleft of the Itk SH3 domain and the adjacent proline-rich region of the Itk TH domain.[17] This structure represents the first characterization of an *intra*molecular SH3 mediated binding event and is proposed to serve a regulatory role in Itk substrate recognition and function. We have more recently characterized an *inter*molecular association between the conserved binding cleft of the Itk SH3 domain and the Itk SH2 domain and have shown that these contacts mediate dimerization of the dual SH3 and SH2 domain-containing fragment of Itk (SH3-SH2).[18] Thus, the *same* conserved SH3 binding pocket within Itk is required for both intramolecular proline recognition and intermolecular association with the SH2 domain to form dimer.

These previously characterized conformational states of Itk are mutually exclusive and therefore raise questions about the structure of the region surrounding the SH3 domain in full length Itk. To elucidate the respective roles of these two conformational states, we have now examined a larger Itk fragment that contains the SH3 domain and both SH3 ligands: the proline-rich region of the TH domain and the SH2 domain. Our data indicate that for this

larger fragment of Itk, dimer predominates in solution and that the SH2 domain, and not the Itk proline-rich region, occupies the conserved SH3 binding pocket.

Closer inspection of the SH3/SH2 mediated dimerization of Itk has revealed a requirement for a *cis* prolyl imide bond within the Itk SH2 domain.[19] In fact, a single conformationally heterogenous proline residue at position 287 within the Itk SH2 domain mediates not only dimerization, but also phospholigand binding. The P287 *trans* conformation is stabilized by canonical phosphotyrosine ligand binding to the SH2 domain.[19] Furthermore, P287 is required for interaction of the Itk SH2 domain with the peptidyl prolyl isomerase, cyclophilin A, which we have shown regulates Itk catalytic activity both *in vitro* and in Jurkat T cells.[19] We now report that the conformational state of P287 within the SH2 domain also controls ligand binding to the adjacent SH3 domain. Mutation of the conformationally heterogeneous proline to glycine, stabilizing the SH2 domain in the *trans* conformation, eliminates Itk dimerization and promotes formation of the *intra*molecular proline-SH3 interaction. Thus, cellular signaling events that alter the conformational state of P287 within the Itk SH2 domain (phospholigand, cyclophilin A binding or Itk dimerization) may all influence the nature of Itk self-association and therefore regulate the interactions of Itk with endogenous signaling partners in the cell.

## Results

### Intermolecular SH3/SH2 interaction out-competes intramolecular proline binding to the SH3 domain

Using NMR spectroscopy, we examined the extent to which Itk SH3/SH2 mediated dimerization competes with formation of the intramolecular proline-SH3 complex. A uniformly $^{15}N$-labeled fragment of Itk containing the proline-rich region (Pr), SH3, and SH2 domains (denoted Pr-SH3-SH2) was expressed in bacteria and purified (Figure 1a). The heteronuclear single quantum coherence (HSQC) spectrum of Pr-SH3-SH2 was compared to that previously reported for the SH3-SH2 dimer[18] (Figure 1b & c, panels *i* & *ii*) and that of the Pr-SH3 fragment (Figure 1b & c, panel *iii*). For residues within the SH3 binding pocket (representative crosspeaks for W208 and S224 are shown in Figures 1b & c, respectively), the Pr-SH3-SH2 resonance positions are similar to those of SH3-SH2 but are quite dissimilar to those of Pr-SH3. These data suggest that for Itk Pr-SH3-SH2, the chemical environment of residues in the SH3 binding pocket is similar to that of the previously characterized SH3-SH2 fragment.

To fully assess the chemical environment of the entire SH3 domain in the larger Itk constructs, we compared the chemical shift values of the isolated Itk SH3 domain with SH3 domain resonances in the context of larger Itk fragments (SH3-SH2, Pr-SH3-SH2 & Pr-SH3). The $^{1}H$ and $^{15}N$ chemical shift differences between the isolated SH3 domain and the larger fragments ($\Delta\delta_X = \delta_{SH3} - \delta_X$, where X= SH3-SH2, Pr-SH3-SH2 or Pr-SH3) are plotted for each SH3 residue in Figure 2. First, in all cases, the observed chemical shift changes map to the conserved binding pocket of the SH3 domain (Figure 3) in agreement with previous structural work.[17,18] The magnitude and direction of the values of $\Delta\delta$ for Itk SH3-SH2 and Pr-SH3-SH2 are similar (compare Figure 2a&b) yet show distinct differences when compared

to the $\Delta\delta$ values measured for Itk Pr-SH3 (Figure 2c). For example, in addition to the chemical shift perturbations illustrated in Figure 1b & c, HSQC crosspeaks corresponding to Y182 and E228 in the Itk SH3-SH2 and Pr-SH3-SH2 constructs show large shifts with respect to Itk SH3 alone but no change in resonance frequency upon intramolecular proline association. In contrast, the $^{15}N$ resonance of R193 shifts significantly downfield upon intramolecular proline binding but changes minimally upon dimer formation.

Analysis of the SH2 resonances within Itk Pr-SH3-SH2 fragment provides additional evidence for the presence of the SH3/SH2 intermolecular interaction within Pr-SH3-SH2. Chemical shift perturbations for the SH2 domain in the context of Pr-SH3-SH2 are similar in magnitude and direction to those previously described[18] for the shorter Itk SH3-SH2 fragment (data not shown). Chemical shift perturbations observed for both the SH3 and SH2 domains therefore indicate that the SH3 binding pocket in the context of Pr-SH3-SH2 is occupied by the previously defined surface of the SH2 domain.[18] Thus, while both *inter*molecular and *intra*molecular self-association events are possible for the Pr-SH3-SH2 fragment of Itk, the SH3/SH2 intermolecular interaction predominates and formation of the intramolecular proline-SH3 complex is negligible.

**Intramolecular proline-SH3 interaction occurs in a Pr-SH3-SH2 mutant that does not form dimer**

While the purified Pr-SH3-SH2 fragment of Itk preferentially dimerizes in solution in a manner similar to the SH3-SH2 fragment, the *intra*molecular proline-SH3 interaction may in

fact be present in full length Itk and play a regulatory role under conditions that disfavor dimer. To eliminate dimerization *in vitro*, we constructed a mutant of Pr-SH3-SH2 in which the proline at position 287 within the SH2 domain is replaced with glycine (denoted Pr-SH3-SH2*, Figure 1a). The amide bond preceding G287 will adopt only the *trans* conformation. The HSQC spectrum of Pr-SH3-SH2* (Figure 1b & c, panel *iv*) was compared to that of Pr-SH3 (Figure 1b & c, panel *iii*), and to the HSQC spectrum of wild type Pr-SH3-SH2, for which dimer predominates (Figure 1b & c, panel *i*). For both the Ser224 backbone amide resonance and the W208 indole NH resonance, Pr-SH3-SH2* is identical to that of Pr-SH3 and not wild type Pr-SH3-SH2.

A more comprehensive analysis reveals that the $\Delta\delta$ values for all of the SH3 resonances in the context of Pr-SH3-SH2* are nearly identical to those of Pr-SH3 (Figure 2d). The observed chemical shift perturbations indicate that 1) the SH3 binding pocket within Pr-SH3-SH2* is occupied by ligand (Figure 3b) and 2) the residues of the SH3 domain in the shorter Pr-SH3 fragment and the mutant Pr-SH3-SH2* are in a similar chemical environment. Thus, for the Pr-SH3-SH2 fragment, mutation of P287 in the Itk SH2 domain to glycine results in formation of the previously characterized intramolecular proline-SH3 interaction. Moreover, a comparison of HSQC spectra of SH2 in the context of the Pr-SH3-SH2* fragment and the isolated Itk SH2 domain mutant bearing the same P287G mutation reveals no chemical shift differences (data not shown). Therefore, unlike Pr-SH3-SH2, the SH2 domain in the Pr-SH3-SH2* fragment is not involved in interactions that result in the chemical shift perturbations observed for the SH3 domain. Thus, for Pr-SH3-SH2*,

dimerization is abrogated and the proline-rich region contacts the SH3 domain in a manner indistinguishable from that of the Pr-SH3 fragment.

**Further biophysical analysis of Itk Pr-SH3-SH2**

NMR linewidths and self-diffusion coefficients ($D_s$)[20] are two independent means to characterize the aggregation state of a molecular species in solution. Linewidths were measured for the Pr-SH3-SH2 and SH3-SH2 fragments at sample concentrations of 1.2 mM. $^1$H NMR linewidths for Itk Pr-SH3-SH2 and SH3-SH2 are similar and range from 26-43 Hz and 28-33 Hz, respectively. A monomeric mutant of Itk SH3-SH2 (SH3*-SH2, where W208 in the SH3 binding pocket is mutated to lysine) exhibits linewidths in the NMR spectrum that range from 19 to 25 Hz. In addition, NMR linewidths for Pr-SH3-SH2* range from 18-23 Hz, consistent with a monomeric species. Thus, the broader lines observed for both SH3-SH2 and Pr-SH3-SH2 indicate that both fragments self-associate to form higher molecular weight species in solution. NMR diffusion experiments were used previously to determine that the Itk SH3-SH2 fragment is involved in a monomer-dimer equilibrium.[18] The self-diffusion coefficient for Pr-SH3-SH2 ($D_s$=6.78$\pm$0.07x10$^{-7}$cm$^2$/s) corresponds to that reported previously for SH3-SH2 ($D_s$=6.81$\pm$0.07x10$^{-7}$cm$^2$/s) providing additional evidence that Pr-SH3-SH2 dimerizes in solution in a fashion similar to that of SH3-SH2 (Figure 4). Together, chemical shift perturbations, NMR linewidths and diffusion data all support a dimeric structure for Pr-SH3-SH2 where the SH3 binding pocket is occupied by the SH2 domain across the dimer interface. Mutation of the conformationally heterogeneous P287 abrogates

dimerization and permits intramolecular contacts between the proline-rich region and the Itk SH3 domain.

**Monomer/dimer equilibrium of Itk Pr-SH3-SH2 fragment is shifted toward dimer compared to SH3-SH2**

It is notable that the magnitude of the observed chemical shift perturbations and the measured linewidths associated with the Pr-SH3-SH2 fragment are generally larger than those observed for the same fragment lacking the proline-rich region (Itk SH3-SH2). Given the fact that the monomer and dimer species are in fast exchange, the resonance frequency and linewidth of each SH3 residue is a weighted average of the frequencies and linewidths associated with the dimeric and monomeric species.[21] Thus, extent of chemical shift changes corresponds to extent of dimerization and suggests that the monomer-dimer equilibrium for Itk Pr-SH3-SH2 is shifted toward dimer to a greater extent than the shorter SH3-SH2 fragment.

To test the extent to which each Itk fragment dimerizes, we monitored chemical shift perturbations during competitive binding assays with isolated SH3 and SH2 domains. We reasoned that the accessibility of both the SH3 and SH2 domains that comprise SH3-SH2 and Pr-SH3-SH2 should be limited by dimerization of these multidomain-containing Itk fragments. This competing equilibrium should therefore limit interaction with exogenous, singly expressed SH2 or SH3 domains (Figure 5a). In an HSQC spectrum of $^{15}$N-labeled SH3 domain, extensive shifts are observed in a subset of SH3 resonances upon addition of

unlabeled Itk SH2 (Figure 5b). For these same residues, smaller shifts are observed upon addition of unlabeled Itk SH3-SH2 to $^{15}$N-labeled SH3 domain (Figure 5b). Dimerization of Itk SH3-SH2 occludes the SH2 binding surface and restricts binding of the isolated $^{15}$N-labeled SH3 domain. The resonance frequencies for residues of the $^{15}$N-labeled SH3 domain are even closer to those of unbound SH3 domain upon addition of unlabeled Itk Pr-SH3-SH2 (Figure 5b). These data are consistent with increased extent of dimerization for the larger Pr-SH3-SH2 fragment that results in a decrease in the accessibility of the SH2 binding surface and a concomitant decreased interaction of Pr-SH3-SH2 with isolated $^{15}$N-labeled SH3 domain. A parallel analysis using $^{15}$N-labeled Itk SH2 domain mixed with the various multi-domain fragments yields the same result (Figure 5c). The accessibility of both the SH3 binding pocket and the SH2 binding surface to exogenous ligands decreases upon dimerization of the SH3-SH2 and Pr-SH3-SH2 fragments. Moreover, it appears that the amino-terminal extension of the Pr-SH3-SH2 fragment enhances Itk dimerization affinity.

**Btk SH2 domain lacks a conformationally heterogeneous proline residue and does not interact with the Btk SH3 domain**

Bruton's tyrosine kinase (Btk)[22,23] is a member of the Tec kinase family. In analogy to Itk, an intramolecular interaction between the proline-rich region of Btk and the adjacent SH3 domain has been observed.[24] To investigate the possibility that Btk self-associates via the intermolecular SH3/SH2 interaction described for Itk, we first analyzed the isolated Btk SH2 domain. While not strictly conserved in position, a proline residue is present in the CD

loop of the Btk SH2 domain, whereas proline is completely absent from the primary amino acid sequence of the other Tec kinases (Figure 6a). We examined the possibility that the Btk SH2 domain might adopt multiple conformations and tested the extent to which the Btk SH2 domain binds to the Btk SH3 domain. The $^{15}$N-labeled Btk SH2 domain was expressed and purified and yielded an HSQC spectrum typical of a domain that adopts a single, well-defined conformation in solution (Figure 6b). The number of backbone and sidechain NH groups in the Btk SH2 domain (residues 272 to 378) matched the number of crosspeaks in the HSQC spectrum. In contrast to Itk, the Btk SH3 domain does not interact with the Btk SH2 domain (Figure 6c, *ii*). We would predict, based on these results, that the larger Btk SH3-SH2 fragment will not dimerize in solution in a manner similar to the corresponding fragment of Itk. Other studies of the Btk SH3-SH2 fragment, however, have indicated the presence of dimer in solution.[25] Dimerization of Btk SH3-SH2 in that case was attributed to formation of a disulfide bond between two Btk SH2 domains and not to an interaction between the Btk SH3 and SH2 domain. In our current study, we see no evidence for dimerization of the Btk SH2 domain (Figure 6b). Finally, we have observed that the conformationally heterogeneous Itk SH2 domain does not interact with the Btk SH3 domain despite sequence similarity between the Itk and Btk SH3 domains (Figure 6c, *iii*). Thus, potentially important structural differences exist among the Tec family kinases; proline *cis/trans* isomerization, and therefore the *cis* dependent dimerization mechanism, may be unique to Itk.

**Discussion**

Structural correlations between SH2 and SH3 domains have been previously reported for several tyrosine kinases. The linker sequence between the SH3 and SH2 domains of the Src kinase appears to mediate cross-domain communication in response to phosphorylation of the Src carboxy-terminal tail.[26] In addition, hydrogen exchange studies indicate that SH3 and SH2 domain dynamics differ when expressed alone or as part of larger, multi-domain containing protein fragments.[27] We have not yet explored the effect of Itk proline *cis/trans* isomerization on the molecular dynamics of the adjacent SH3 domain, but instead have characterized a dynamic conformational switch within the SH2 domain that dictates ligand recognition and accessibility of the neighboring SH3 domain. Whether molecular motions or molecular recognition are coupled phenomena, the fact that SH3 and SH2 domains are often consecutive in primary amino acid sequence may be an important factor in the control of cellular signaling specificity.

We have now determined that proline *cis/trans* isomerization within the Itk SH2 domain governs the nature of SH3-mediated Itk self-association. The *cis* prolyl imide bond at position 287 within the Itk SH2 domain is required for dimerization through *inter*molecular contacts to an opposing SH3 binding pocket (Figure 7a).[19] For the *trans* conformation of this peptide bond, dimerization is abrogated and the adjacent SH3 binding pocket is occupied in an *intra*molecular fashion by the proline-rich region of the TH domain (Figure 7b). Thus, *cis/trans* isomerization around the P287 imide bond within the Itk SH2 domain serves as a molecular switch that controls ligand occupancy of the adjacent SH3 domain and therefore the

quaternary structure of Itk. In multi-domain containing fragments of Itk in which competing equilibria between intra- and intermolecular self-association is possible, intermolecular self-association appears to be favored over the intramolecular proline-SH3 interaction.

Our data now clarify the interpretation of biochemical data that was reported with the structure of the intramolecular Itk proline-SH3 complex.[17] In that report, binding of an SH3 ligand, Sam-68, to a panel of recombinant SH3-containing Itk fragments was assayed by immunoblotting. First, it was observed that for Itk constructs containing only the SH3 domain and the adjacent proline-rich region, Sam-68 out-competed the intramolecular proline-SH3 interaction and associated directly with the Itk SH3 domain. In complete agreement with data presented here in Figure 5, the accessibility of the Itk SH3 binding pocket diminished significantly for larger Itk constructs containing the proline-rich region, the SH3 domain *and* the SH2 domain. In fact, Itk fragments spanning the TH domain through the SH2 domain (TH-Pr-SH3-SH2) did not associate with Sam-68 to a detectable level. Given the lack of knowledge regarding the SH3/SH2 mediated dimerization of Itk at the time, these data were interpreted to indicate that the *intra*molecular interaction between the SH3 domain and the proline-rich region is stabilized by the presence of the SH2 domain.[17] Our current data now reveal that reduced accessibility of the Itk SH3 domain in multi-domain containing constructs is the result of dimerization and not a stable intramolecular proline-SH3 interaction.

It is interesting to note that the affinity of the SH3/SH2 mediated dimerization appears to increase in the Pr-SH3-SH2 fragment. This is perhaps unexpected given that direct competition for the SH3 binding pocket between the intramolecular proline ligand and

intermolecular SH2 binding should lead to a decrease in the affinity of dimerization. One possible explanation for the observed increase in affinity is that the proline-rich region is actively involved in stabilizing dimer, either by stabilizing the *cis* SH2 conformer or by direct contacts across the dimer interface. In fact, we have observed chemical shift perturbations for a subset of SH2 resonances in the Pr-SH3-SH2 fragment that are not observed in the SH3-SH2 fragment (unpublished results, K.N.B & A.H.A.). These preliminary data indicate that, like the Itk SH3 domain, the proline-rich region may serve dual structural roles depending on the quaternary structure of Itk.

The significance of the different quaternary structures of Itk in T cell signaling remains elusive. We have recently reported a direct regulatory interaction between the Itk SH2 domain and the peptidyl prolyl isomerase, cyclophilin A.[19] The cyclophilin A-Itk interaction requires P287 in the Itk SH2 domain, maps to the surface of the SH2 domain required for Itk dimerization, and inhibits Itk catalytic activity.[19] We therefore predict that dimerization will be prevented in the inactive, cyclophilin A-bound state of Itk. The P287G mutant described here therefore serves as a model for the proposed monomeric, down-regulated form of Itk. Importantly, impedance of Itk dimerization in this mutant leads to formation of the *intra*molecular interaction between the SH3 binding pocket and the adjacent proline-rich ligand of the TH domain. Thus, in the context of the inactive Itk kinase, the intramolecular proline-SH3 interaction may play an important regulatory role in limiting the ability of the Itk SH3 domain to interact with exogenous signaling partners. Itk dimerization may be a mechanism by which Itk is activated. Itk dimerization through SH3/SH2 mediated

interactions would facilitate displacement of cyclophilin from the Itk SH2 domain and removal of the proline-rich region from the SH3 binding pocket. Whether the dimer is retained in fully active Itk kinase or whether subsequent activation steps lead to dissociation of the dimer remains a question. Nevertheless, each of the quaternary structures of Itk appears to be associated with a discrete conformation around the imide bond preceding P287. Certainly, the conformational switch afforded by proline *cis/trans* isomerization within Itk is an important component of the Itk regulatory apparatus.

The observation that the Btk SH2 domain does not exhibit conformational heterogeneity, combined with the absence of a direct Btk SH3/SH2 interaction, indicates that the apparently closely related Tec kinases differ significantly at a structural level and therefore in the mechanisms that control ligand recognition and catalytic activity.[28] Previous structural work on the Btk and Tec kinases has revealed a propensity for dimerization through canonical proline-SH3 interactions instead of the SH3/SH2 intermolecular interaction discussed here for Itk.[24,29,30] Dimerization of the Tec kinases may therefore serve a regulatory role in general, yet the molecular details of the dimer interface for each of the Tec kinases may differ dramatically. The observation that the *cis* Itk SH2 surface is recognized by the Itk SH3 domain and not the SH3 domain of Btk is noteworthy (Figure 5). The Itk and Btk SH3 domains are 52% identical and differ at 7 of the 20 residues at the SH3-SH2 dimer interface. Some of the amino acid differences in the Itk and Btk SH3 binding pockets may be significant in terms of steric and electrostatic contributions to ligand recognition. For example, the alanine at position 221 in Itk corresponds to isoleucine in Btk, Cys194 and

Glu205 in Itk correspond to lysine and asparagine, respectively, in the Btk sequence. Thus, the specificity of the Itk SH3/SH2 interaction must involve unique structural features of both the SH3 and SH2 domains. The precise nature of the specificity-determining elements for this interaction and the role of dimerization and proline isomerization in regulating Tec family signaling will be clarified as additional structural and biochemical work is completed.

## Materials and Methods

### Protein expression and NMR Spectroscopy.

Full-length Itk and Btk cDNA used for PCR amplification were gifts from Leslie J. Berg (University of Massachusetts Medical School) and Xin-Yun Huang (Cornell University Medical College), respectively. Domain boundaries for the multi-domain-containing Itk constructs are as follows (amino acid numbering corresponds to full length Itk[11]: Itk Pr-SH3 (154-232), Pr-SH3-SH2 and Pr-SH3-SH2* (154-338), SH3-SH2 and SH3*-SH2(W208K) (171-338). Protein samples used in these NMR studies were expressed and purified as described previously.[18] Due to low levels of soluble expressed protein, the Itk Pr-SH3-SH2* mutant was extracted from inclusion bodies using 1.5% sarcosyl (Fisher). Subsequent purification steps remained the same although it should be noted that the Pr-SH3-SH2* mutant was less stable following purification. The sample precipitated before more extensive characterization (such as NMR diffusion experiments) could be carried out. All NMR spectra were recorded at 25°C on a Bruker DRX500 spectrometer operating at a $^{1}H$ frequency of 499.867 MHz. A gradient-enhanced HSQC experiment with minimal water

saturation was used for all $^{1}H$-$^{15}N$ correlation experiments.[31] The self-diffusion coefficient $D_S$ for the Itk Pr-SH3-SH2 fragment was measured as described previously[18] by use of a longitudinal encode-decode (LED) gradient experiment.[20,32]

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**Figure 1.** (a) Domain structure of Itk and the corresponding SH3-containing fragments (Pr-SH3-SH2, SH3-SH2, Pr-SH3, SH3, and Pr-SH3-SH2*). The proline-rich region (Pr) is part of the TH domain and is indicated by a small rectangle N-terminal to the SH3 domain. The relative positions of W208 and S224 in the SH3 domain and P287 in the SH2 domain are indicated. (b) HSQC spectra corresponding to four different Itk fragments: (*i*) Itk Pr-SH3-SH2 (1.2mM), (*ii*) Itk SH3-SH2 (1.2 mM), (*iii*) Itk Pr-SH3 (1.25 mM), and (*iv*) Itk Pr-SH3-SH2* (P287G mutation, 1 mM). The region shown includes the indole NH of W208 and W209 in the Itk SH3 domain. (c) For (*i-iv*), the backbone resonance of S224 is shown for the Pr-SH3-SH2, SH3-SH2, Pr-SH3 and Pr-SH3-SH2* fragments, respectively. (*i, ii, iv)* the amide backbone resonance for E269 of the SH2 domain is labeled. For both (b) and (c), the HSQC spectrum of the isolated Itk SH3 domain, 1.2mM (gray) is superimposed in each panel. The arrows indicate the extent and direction of chemical shift changes that are observed between SH3 alone and the larger Itk constructs. Assignment of resonances in the HSQC spectra of the large fragments was accomplished by comparison to previously assigned spectra of the isolated SH3 and SH2 domains.[17,18]

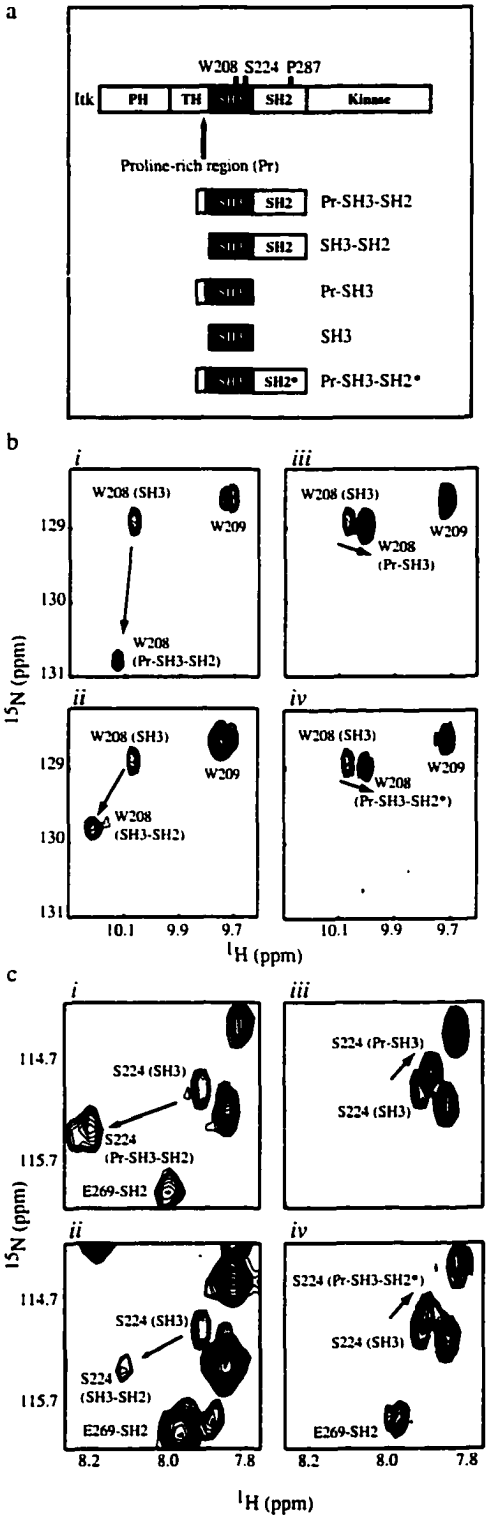

a
W208 S224 P287
Itk
PH
TH
SH2
Kinase
Proline-rich region (Pr)
Pr-SH3-SH2
SH3-SH2
Pr-SH3
SH3
Pr-SH3-SH2*
b
i
iii
ii
iv
W208 (SH3)
W209
W208 (Pr-SH3-SH2)
W208 (Pr-SH3)
W208 (SH3-SH2)
W208 (Pr-SH3-SH2*)
129
130
131
15N (ppm)
10.1
9.9
9.7
1H (ppm)
c
114.7
115.7
S224 (SH3)
S224 (Pr-SH3-SH2)
E269-SH2
S224 (Pr-SH3)
S224 (SH3-SH2)
S224 (Pr-SH3-SH2*)
8.2
8.0
7.8

**Figure 2.** Chemical shift perturbation analysis of the SH3 domain residues in four different Itk constructs. The plotted values correspond to chemical shift differences ($\Delta\delta$) between the isolated SH3 domain and the larger SH3-containing fragment ($\Delta\delta_X = \delta_{SH3} - \delta_X$, where X= SH3-SH2, Pr-SH3-SH2, Pr-SH3 or Pr-SH3-SH2*), (a) Itk SH3-SH2, (b) Itk Pr-SH3-SH2, (c) Itk Pr-SH3, and (d) Itk Pr-SH3-SH2*. $\Delta\delta$ values for $^{15}N$ (open bars) and $^{1}H$ (closed bars) resonances are illustrated. The plus sign (+) represents residues that change in resonance frequency relative to the SH3 domain alone; however, the change in chemical shift or line broadening occurred to an extent that the resonances could no longer be unambiguously assigned. For the Pr-SH3-SH2 fragment in (b) the sidechain $^{15}N$ resonance shift for W208 is greater than 1 ppm as indicated by the break in the bar. Numbering of the SH3 residues corresponding to full length Itk is provided in (a). Bold residues are referred to specifically in the text.

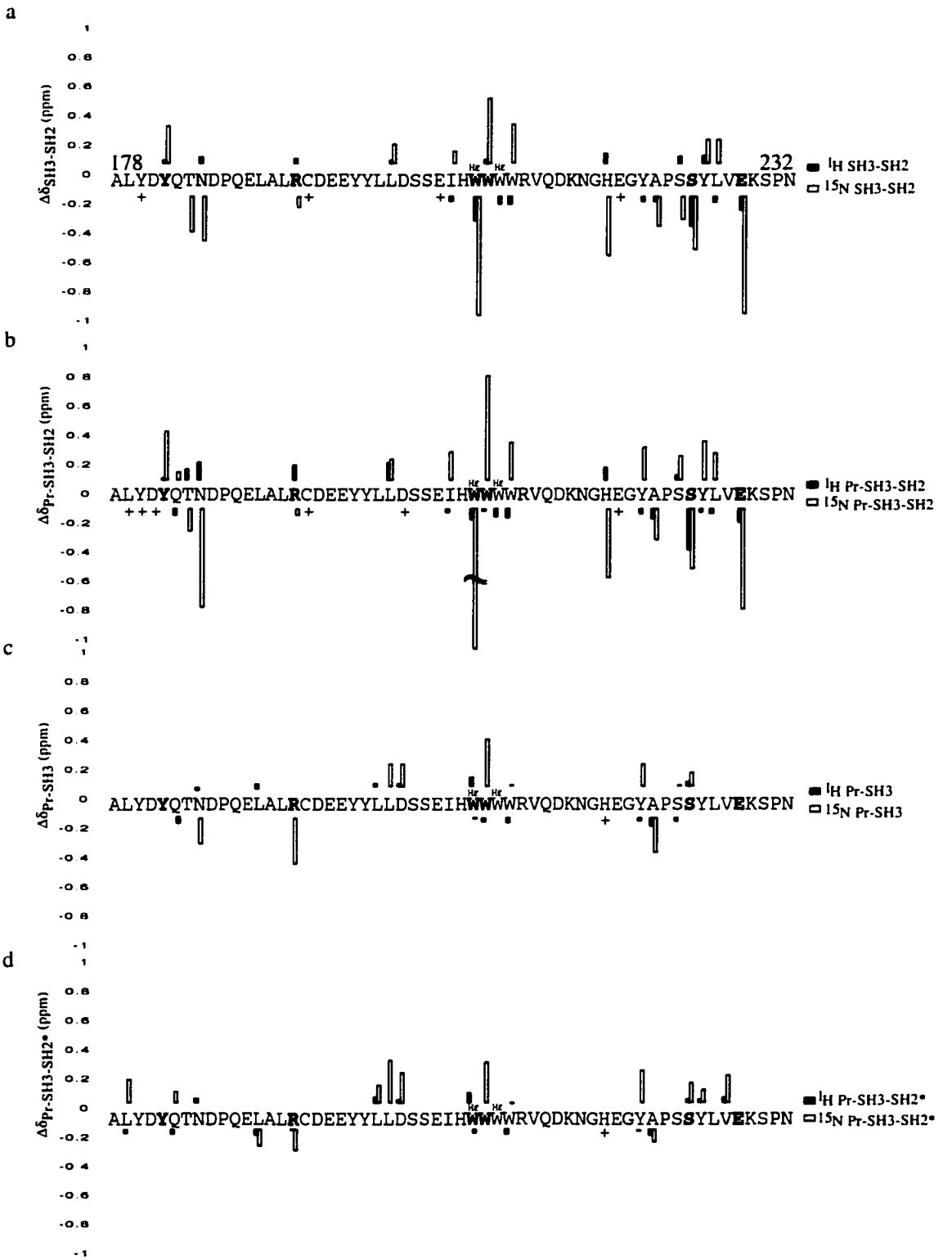

a
$\Delta\delta_{SH3-SH2}$ (ppm)
178
232
ALYDYQTNDPQELALRCDEEYYLLDSSEIHWWWWRVQDKNGHEGYAPSSYLVEKSPN
$^1$H SH3-SH2
$^{15}$N SH3-SH2
b
$\Delta\delta_{Pr-SH3-SH2}$ (ppm)
ALYDYQTNDPQELALRCDEEYYLLDSSEIHWWWWRVQDKNGHEGYAPSSYLVEKSPN
$^1$H Pr-SH3-SH2
$^{15}$N Pr-SH3-SH2
c
$\Delta\delta_{Pr-SH3}$ (ppm)
ALYDYQTNDPQELALRCDEEYYLLDSSEIHWWWWRVQDKNGHEGYAPSSYLVEKSPN
$^1$H Pr-SH3
$^{15}$N Pr-SH3
d
$\Delta\delta_{Pr-SH3-SH2*}$ (ppm)
ALYDYQTNDPQELALRCDEEYYLLDSSEIHWWWWRVQDKNGHEGYAPSSYLVEKSPN
$^1$H Pr-SH3-SH2*
$^{15}$N Pr-SH3-SH2*

**Figure 3**. Three dimensional solution structure of the Itk SH3 domain[17] illustrating the surface residues involved in binding to either the proline-rich region (a) or the SH2 domain (b). The well-defined ligand-binding site of the SH3 domain mediates both intramolecular and intermolecular interactions of the SH3 domain with the proline-rich region and the SH2 domain, respectively. (a) Side chains highlighted in red correspond to those that exhibit a chemical shift change upon dimerization of both SH3-SH2 and Pr-SH3-SH2. The yellow residue is affected by self-association of Pr-SH3-SH2 and not the shorter SH3-SH2 fragment. (b) Side chains highlighted in red correspond to those that exhibit chemical shift perturbations upon formation of the intramolecular proline-SH3 interaction (Pr-SH3 and Pr-SH3-SH2*). Yellow denotes side chains that exhibit chemical shift perturbations in Pr-SH3-SH2* and not Pr-SH3.

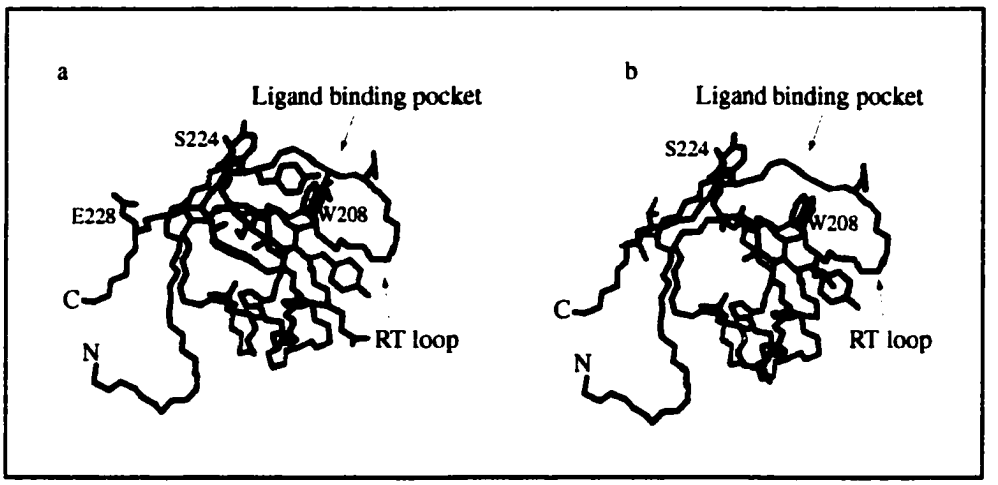

a
Ligand binding pocket
S224
E228
W208
C
N
RT loop
b
Ligand binding pocket
S224
W208
C
N
RT loop

**Figure 4.** NMR self-diffusion data for Itk Pr-SH3-SH2 (▼), Itk SH3-SH2 (●) and the monomeric mutant Itk SH3*-SH2(W208K) (O). The data are the average values of normalized peak integrals obtained from four replicate experiments. Definition of variables and method for determination of the reported self-diffusion coefficients ($D_s$) was carried out as described previously.[18] The data shown correspond to the last nine points of those shown in Figure 6 of reference 18, i.e., the range of the data for which the signal decay corresponding to the monomeric and dimeric species diverge. The error bars represent three times the standard deviation of the data from the individual experiments.

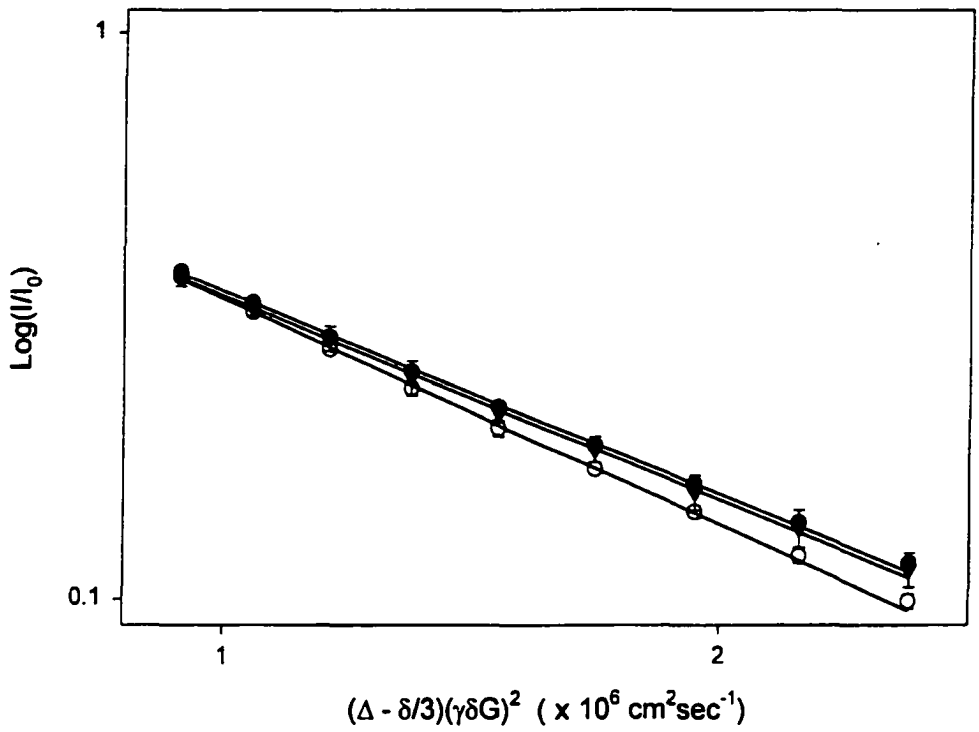

1
0.1
Log(I/I0)
1
2
(Δ - δ/3)(γδG)2 ( x 10^6 cm^2sec^-1)

**Figure 5**. (a) An example of an equilibrium model for the competitive binding experiments shown in (b). $^{15}$N-labeled SH3 domain (circled) is mixed with unlabeled (NMR inactive) SH3-SH2. Two competing equilibria in fast exchange are possible: A) formation of the homodimer of SH3-SH2 plus unbound $^{15}$N-SH3 which will resonate at the same frequency as $^{15}$N-SH3 alone or B) formation of the mixed dimer of $^{15}$N-SH3 and SH3-SH2 for which resonance frequencies of the $^{15}$N-labeled SH3 domain reflect binding to the SH2 domain. In each case the species in solution that gives rise to an NMR signal is circled. (b) An overlay of a select region of HSQC spectra illustrating the crosspeaks corresponding to Glu228 in the Itk SH3 domain in the presence of three different Itk fragments. $^{15}$N-labeled Itk SH3 alone (1.7 mM) (*i*) was independently mixed with equimolar, unlabeled Itk SH2 (*ii*), Itk SH3-SH2 (*iii*), and Itk Pr-SH3-SH2 (*iv*). (c) An overlay of a select region of HSQC spectra that includes the crosspeaks corresponding to Thr331 in the Itk SH2 domain in the presence of three different Itk fragments. $^{15}$N-labeled Itk SH2 alone (1.7 mM) (*i*) was independently mixed with equimolar, unlabeled Itk SH3(*ii*), Itk SH3-SH2(*iii*) and Itk Pr-SH3-SH2(*iv*). The position of the crosspeak relative to isolated SH3 or SH2 domain indicates the extent to which the isolated domain interacts with the larger fragment. The smaller chemical shift differences indicate a less extensive interaction and a higher fraction of unbound, labeled domain.

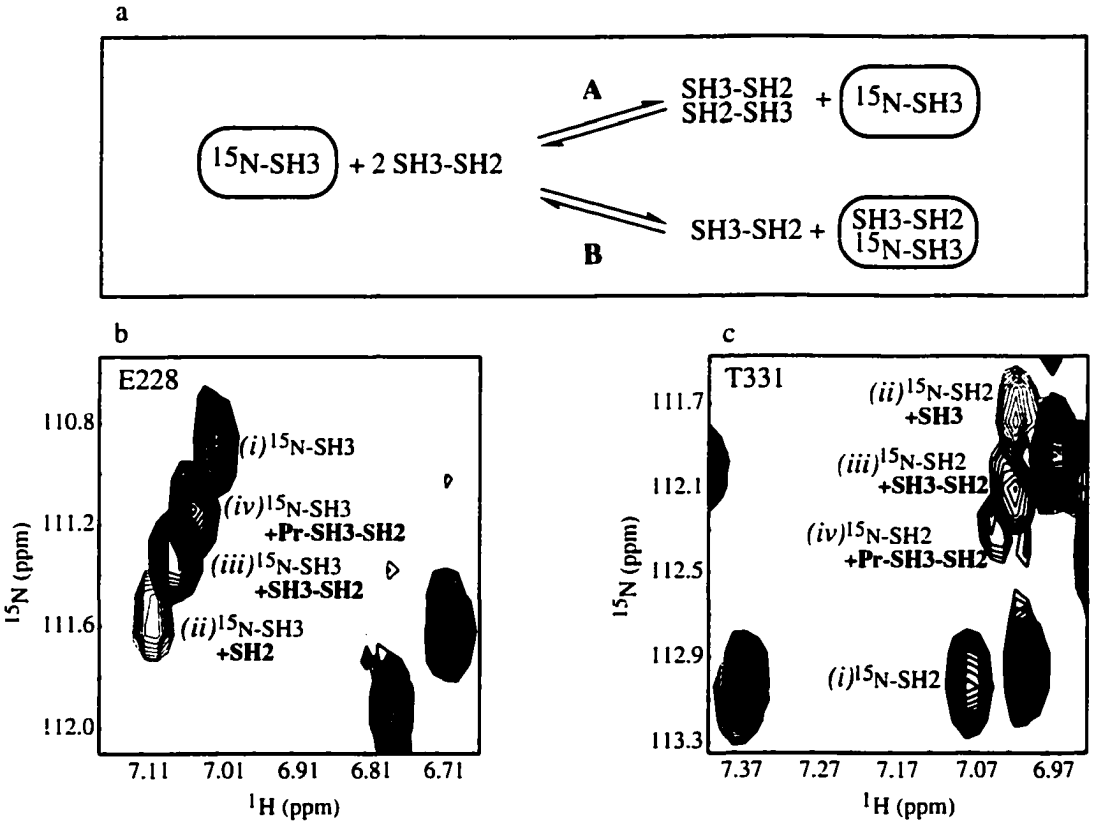

a
A
B
15N-SH3 + 2 SH3-SH2
SH3-SH2
SH2-SH3 + 15N-SH3
SH3-SH2 + SH3-SH2
15N-SH3
b
E228
(i)15N-SH3
(iv)15N-SH3
+Pr-SH3-SH2
(iii)15N-SH3
+SH3-SH2
(ii)15N-SH3
+SH2
110.8
111.2
111.6
112.0
15N (ppm)
7.11
7.01
6.91
6.81
6.71
1H (ppm)
c
T331
(ii)15N-SH2
+SH3
(iii)15N-SH2
+SH3-SH2
(iv)15N-SH2
+Pr-SH3-SH2
(i)15N-SH2
111.7
112.1
112.5
112.9
113.3
15N (ppm)
7.37
7.27
7.17
7.07
6.97
1H (ppm)

**Figure 6.** (a) A sequence alignment generated using Clustalx[33] of the CD loop within the SH2 domain from the five Tec kinases. An asterisk indicates the position of P287 in the Itk SH2 domain. Sequence accession numbers are: Itk, A47333; Btk, P35991; Rlk, A55631; Tec, T01380; Bmx, AAB47770.1. (b) Entire HSQC spectrum of purified, recombinant Btk SH2 domain. (c) (*i*) A select region of the $^{15}$N Itk SH3 HSQC spectrum alone (black) and in the presence of equimolar Itk SH2 (gray) illustrating chemical shift changes due to the Itk SH3/SH2 interaction. (*ii*) $^{15}$N Btk SH3 HSQC spectrum alone (black) and in the presence of equimolar Btk SH2 (gray). The region shown includes the indole NH of W251 (major form), W251m (minor form) and W252 in the Btk SH3 domain-binding pocket. (*iii*) Same region as (*ii*). $^{15}$N Btk SH3 HSQC spectrum alone (black) and in the presence of equimolar Itk SH2 domain (gray). No chemical shift perturbations are observed for the Btk titrations.

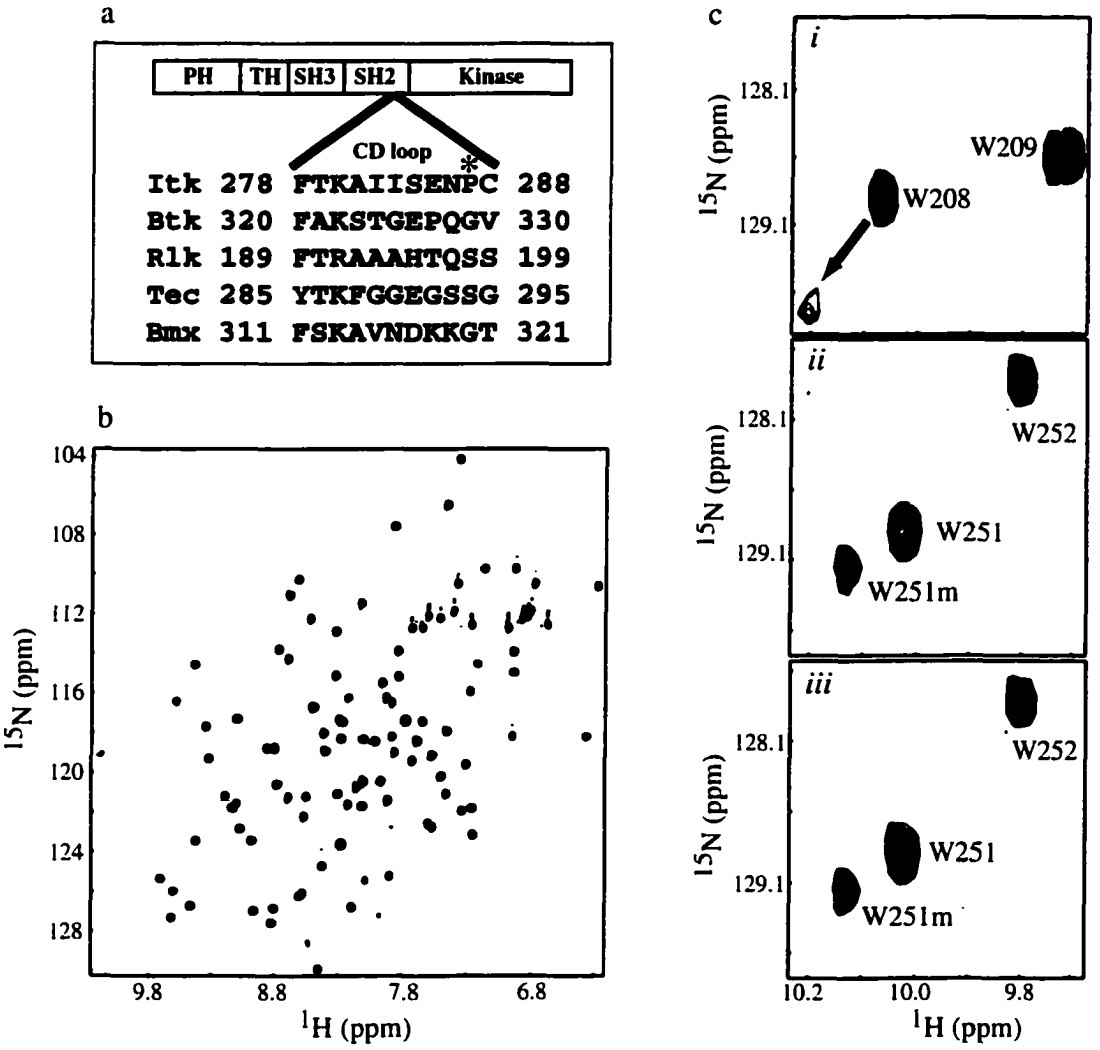
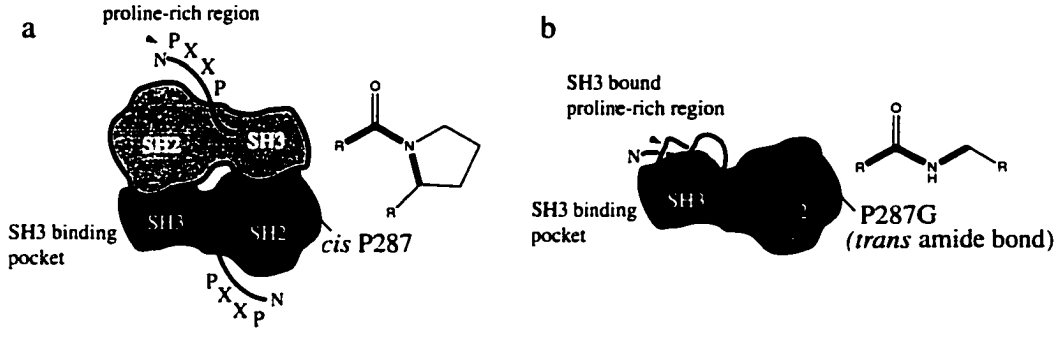

a
PH
TH
SH3
SH2
Kinase
CD loop
Itk 278 FTKAIISENPC 288
Btk 320 FAKSTGEPQGV 330
Rlk 189 FTRAAAHTQSS 199
Tec 285 YTKFGGEGSSG 295
Bmx 311 FSKAVNDKKGT 321
b
104
108
112
116
120
124
128
15N (ppm)
9.8
8.8
7.8
6.8
1H (ppm)
c
i
128.1
129.1
15N (ppm)
W209
W208
ii
128.1
129.1
15N (ppm)
W252
W251
W251m
iii
128.1
129.1
15N (ppm)
W252
W251
W251m
10.2
10.0
9.8
1H (ppm)

**Figure 7.** (a) Model of the Itk Pr-SH3-SH2 dimer. Intermolecular interaction between the Itk SH3 and SH2 domains requires the *cis* conformation of the imide bond preceding P287. (b) Mutation of P287 to glycine eliminates the possibility that the 286-287 amide bond can adopt the *cis* conformation and results in formation of the intramolecular proline-SH3 interaction.


a
proline-rich region
P
X
X
P
N
SH2
SH3
SH3
SH2
SH3 binding
pocket
cis P287
P
X
X
P
N
b
SH3 bound
proline-rich region
N
SH3 binding
pocket
P287G
(trans amide bond)

# CHAPTER 5. GENERAL SUMMARY AND CONCLUSIONS

This dissertation provides insight into role of the SH3 and SH2 domains of interleukin-2 tyrosine kinase (Itk) in regulating protein-protein interactions and catalytic activity, thereby extending the understanding of these domains in mediating protein-tyrosine kinase function. A proline *cis/trans* isomerization event has been identified in the Itk SH2 domain using nuclear magnetic resonance spectroscopy (NMR) (1). This slow exchange event causes the SH2 domain to adopt two distinct conformations in solution, a *trans* proline-containing conformer and a *cis* proline-containing conformer. Larger fragments of Itk, e.g. SH3SH2 and PrSH3SH2, preserve this conformational heterogeneity (2, 3). Likewise, *in vitro* ligand binding assays reveal the presence of both the *cis* and *trans* conformers in full length Itk (1). The *trans* confomer appears to be two times more populated than the *cis* conformer in small fragments of Itk; however, in the context of full length Itk, this ratio may be different.

The *cis* and *trans* conformers display distinct ligand binding specificities (1). The canonical phosphotyrosine-containing SH2 ligand, through the well-characterized SH2 domain ligand binding surface, preferentially binds the *trans* conformer. In contrast, the Itk SH3 domain, a novel ligand binding partner, preferentially binds to the *cis* conformer. The SH3-SH2 interaction is dependent upon the conformationally heterogenous proline residue in the Itk SH2 domain, and reveals a newly defined ligand binding surface on the Itk SH2 domain (2). The SH3-SH2 interaction surface encompasses four main loops in the SH2 domain, the

AB, BG, EF and CD loops; the EF and BG loops also form part of the phosphotyrosine specificity pocket. The SH3-SH2 interaction surface maps out to the canonical ligand binding pocket on the SH3 domain, known to bind proline–rich sequences containing the PXXP motif. However, a PXXP motif is not found within the region of the SH2 domain involved in the SH3-SH2 interaction, nor is a phosphotyrosine-containing (pTyr) sequence present within the SH3 domain. Thus, the SH3-SH2 interaction reveals novel ligand binding capacities of both domains.

The Itk SH3 and SH2 domains were not able to bind SH2 and SH3 domains from other proteins; therefore, the Itk SH3-SH2 interaction appears to be specific (2, 3). In addition, the Itk SH2 domain did not interact with the SH3 domain of the closely related Tec family kinase Btk. This result provides evidence that Tec family kinase members may differ significantly on a structural level. The unique ligand specificity of the Itk SH3 and SH2 domains may be necessary to regulate explicit interactions with other proteins, although it remains to be determined if Itk is capable of interacting with other signaling partners through an SH3-SH2 interaction.

The Itk SH3SH2 dual domain-containing fragment and the larger PrSH3SH2 fragment self-associate in solution through the SH3-SH2 intermolecular interaction (2, 3). Dimerization of the SH3SH2 and PrSH3SH2 fragments limits the accessibility of the SH2 domain to ligands, e.g. single Itk SH3 domain and pTyr peptide, and the SH3 domain to ligands, e.g. single Itk SH2 domain and polyproline peptide, based on binding competition experiments. The SH3 and SH2 domain ligand binding sites appear to become less accessible

as the Itk fragments become increasingly larger, suggesting that full-length Itk may have a propensity to dimerize. It is therefore possible that Itk dimerization *in vivo* is involved in regulating Itk activity. Membrane localization of Itk in response to antigen receptor stimulation may increase the local concentration of Itk, resulting in Itk dimerization. Although the activation state of the putative Itk dimer remains unknown, it may be required for SH3 domain autophosphorylation in *trans*, which leads to Itk activation (4-6). Alternatively, dimerization may be a means of controlling Itk function by hindering the accessibility of the regulatory domains, thereby preventing interactions with other signaling molecules, providing a plausible mechanism for both activation and inactivation.

The Itk SH3-SH2 mediated self-association is in contrast to the mechanism of self-association for the other Tec family kinases (7-9). Btk and Tec have been shown to dimerize *via* an intermolecular proline-SH3 domain interaction. In fact, the Btk SH3 domain did not appear to have any affinity for its own SH2 domain (3). Perhaps the different mechanisms of self-association suggest that the Itk SH2 domain may play a principal role in regulating the function of Itk compared to the role of the SH2 domains in the other Tec family kinases. Regardless of the mode of self-association, all of the Tec family members studied to date appear to dimerize, and dimerization may be a means of regulating catalytic activity within the Tec family. Thus, further assessment of the effects of dimerization on all the Tec family kinases is necessary before one can determine the importance of self-association.

In addition to mediating the Itk SH3-SH2 interaction, the conformationally heterogenous proline residue is required for the recognition of the Itk SH2 as a substrate for

the peptidyl-prolyl isomerase (PPIase) cyclophilin A (1). Cyclophilin A (CyP A) appears to increase the exchange rate between the *cis* and *trans* proline conformers at substoichiometric concentrations as determined by NMR. The acceleration in the exchange rate appears to be specific for CyP A since there was no evidence of an increase in the exchange rate between the conformers in the presence of the PPIase FK-506 binding protein (FKBP).

CyP A appears to be an inhibitor of Itk catalytic activity *in vitro* (1). The level of Itk autophosphorylation was significantly lower in the presence of CyP A, as measured using an *in vitro* kinase assay. The inhibitory effect of CyP A on Itk autophosphorylation levels was diminished when CyP A was pretreated with the cyclophilin inhibitor cyclosporin A (CsA) prior to the *in vitro* kinase assay.

CyP A also seems to regulate Itk phosphorylation levels in Jurkat T cells (1). Itk phosphorylation levels were higher after anti-CD3 stimulation in Jurkat cells pretreated with CsA than in untreated cells. In other words, CsA acts to activate Itk, likely through inhibiting the catalytic site of Cyp A, which prevents Cyp A inhibition of Itk. In addition, Itk phosphorylation levels decreased with increasing amounts of co-immunoprecipitated CyP A. Thus, providing further evidence that CsA treatment led to increases in Itk phosphorylation by alleviating CyP A inhibition of Itk. Consistent with this analysis was the observation that following anti-CD3 stimulation, increases in the phosphorylation levels of the downstream signaling partner of Itk, phospholipase Cγ1 (PLC γ1), paralleled increases in Itk phosphorylation levels in cells pretreated with CsA. In contrast, the phosphorylation

levels after anti-CD3 stimulation of the 70 kD zeta chain associated protein (Zap 70), an upstream signaling partner of Itk, did not increase with CsA pretreatment compared to untreated cells. Similar to the specificity observed by NMR, Itk phosphorylation levels remained unaffected after anti-CD3 stimulation in cells pretreated with the inhibitor of FKBP, FK-506, compared to untreated cells.

The effect of CyP A on Itk phosphorylation in Jurkat T cells appears to be mediated by the conformationally heterogenous Itk SH2 domain (1). Competitive binding experiments with ligands that target the SH2 domain were able to disrupt CyP A co-immunoprecipitation. Thus, the Itk-CyP A interaction will likely disrupt Itk SH3-SH2 mediated dimerization, and may stabilize a down-regulated, monomeric form of Itk. The conformational state of CyP A bound Itk has not been determined, although one can speculate that when the SH2 domain is unavailable for dimerization the intramolecular proline-SH3 domain interaction, which is prevented in larger fragments of Itk as a result of self-association, may occur (2, 3, 10). Analogous to CyP A bound Itk, it was observed that the proline-rich region could bind intramolecularly to a mutant PrSH3SH2(P287G) fragment in which the conformationally hetergenous proline was mutated to a glycine, and the protein therefore was unable to self-associate (3). Consequently, both the SH3 and SH2 domain ligand binding sites may be unavailable in CyP A bound Itk, providing a possible mechanism for Itk regulation.

Currently, the precise mechanism of CyP A regulation of Itk activity remains unclear. CyP A may be acting as a catalyst to increase the rate of *cis/trans* isomerization, allowing more rapid responses to ligand binding events, or may be acting as a stable binding partner,

occluding ligand binding sites. T cell receptor signaling events occur within seconds of contact with an antigen-receptor presenting cell, leading to rapid increases in intracellular protein phosphorylation levels (11-13). CyP A, as a catalyst, may be necessary to accelerate Itk *cis/trans* isomerization in order for Itk to respond rapidly to antigen receptor stimulation. CyP A, as a binding partner, may regulate ligand binding interactions to both the SH3 and SH2 domains, as well as inhibit Itk catalytic activity. Nevertheless, the identification of the Itk-CyP A interaction provides the first example of CyP A in regulating the catalytic activity of a non-receptor tyrosine kinase. Not only does this allow us to propose a new mode of kinase regulation but also provides a cellular role for CyP A in mediating T cell signaling events. In broader terms, identification of the Itk SH2 domain as a substrate for CyP A bestows the opportunity to study the mechanistic details of a PPIase on a folded protein substrate in solution.

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